001 [Oral 049]

The Human *SPINK5* Gene Encodes Multiple LEKTI Isoforms Derived from Alternative RNA Processing

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SPINK5, the mutated gene in the severe autosomal recessive ichthyosiform skin disorder, Netherton syndrome, encodes the putative serine protease inhibitor LEKTI (lympho-epithelial Kazal-type related inhibitor). Strongly expressed in the most differentiated viable layers of all stratified epithelia, this protein is thought to be involved in different biological pathways relevant to epithelial tissue homeostasis and possibly also to inflammation and anti-microbial defense. LEKTI, as deduced from the nucleotide sequence of the cloned cDNA, consists of 1064 amino acids organized into 15 potential inhibitory Kazal-type domains (D1-D15) preceded by a signal peptide, and is predicted to weight ~120 kDa. However, we recently reported the detection of two N-glycosylated precursor proteins (~145 and ~125 kDa) and three C-terminal proteolytic fragments (~68, 65 and 42 kDa) in differentiated normal human keratinocytes (HK). Here, we show that, following alternative processing of its pre-mRNA, *SPINK5* generates three classes of transcripts, each differing in the 3' end portion of the coding region. In addition to the previously described 3.7 kb mRNA, termed full-length, a shorter transcript of ~3 kb generated by the recognition of an intronic polyadenylation signal, and a transcript carrying the insertion of a 90 bp cryptic exon located within intron 28 (exon 28 bis) were identified by screening a HK cDNA library. In keeping with *SPINK5*/LEKTI expression in the uppermost differentiated layers of the epidermis, the expression level of all *SPINK5* transcripts significantly increases as HK differentiate *in vitro*. However, the ratio among the different transcripts progressively changes during keratinocyte differentiation thus suggesting that the alternative processing of the *SPINK5* pre-mRNA is a differentiation-regulated process. Still, dot blot and real-time RT-PCR analysis of RNA samples from various human tissues showed that the three *SPINK5* transcripts display a diverse tissue distribution. Sharing the same transcription start site, the *SPINK5* mRNAs are expected to result in three LEKTI isoforms: the 1064 amino acids full-length protein containing the 15 inhibitory domains, a shorter form lacking the last two domains (D14 and D15), and a longer form carrying the in-frame insertion of 30 residues within the linker region between D13 and D14. Accordingly, in addition to the previously described ~145 and 125 kDa LEKTI precursor proteins, a third isoform of ~148 kDa could be detected in differentiated normal HK. Furthermore, a polyclonal antibody raised against the protein domains D14-D15 recognized only the ~148 and 145 kDa LEKTI isoforms indicating that the ~125 kDa polypeptide represents the translation product of the ~3 kb *SPINK5* transcript. These data suggest that the transcriptional and post transcriptional mechanisms regulate, in human, LEKTI isoforms expression. Since the bioactive forms of LEKTI appear to derive from the intracellular cleavage of the precursor proteins, we hypothesize that the LEKTI bioactive fragments may have a different target specificity.

002 [Oral 007]

Characterization of Sequences Important for the Interaction of Desmoplakin (DP) and Plectin (PL) with Desmin. Towards Understanding of the Molecular Basis of Inherited Skin Disorders Associated with Myopathies (MD) and Cardiomyopathies (CM)

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DP and PL, versatile cytolinkers of the plakin family, connect the intermediate filament (IF) system to other cytoskeletal networks and/or distinct sites at the plasma membrane, such as desmosomes and hemidesmosomes. Inherited defects of the DP or PL gene result in epidermolysis bullosa (EB) or palmoplantar keratoderma (PPK) in association with MD and/or CM. Despite their homology, the COOH termini of DP and PL have the ability to selectively bind to distinct IF proteins. Although their association with epidermal keratins has been previously characterized, their potential to interact with desmin has been poorly investigated. We have here studied sequences within their tail important for binding to desmin by yeast two-hybrid, cell transfection and overlay assays.

Our results indicate that: 1) DP interacts with desmin via a region encompassing the linker region between the B and C subdomains. However, the COOH-terminal extremity and the B and C subdomain also appear to contribute to binding; 2) a DP mutant carrying a substitution in the tail, DP-BC^{G2375R}, identified in a patient with PPK and CM, has lost the ability to interact with desmin; 3) the B subdomain and linker region of PL can also associate with desmin, whereas the C subdomain and COOH extremity do not. Notably, a truncated plectin molecule lacking the last 35-amino acids of the tail cause disorganization of the desmin cytoskeleton in a patient with EB-MD, suggesting that the COOH terminal portion also support binding; 4) the interaction of DP and PL with vimentin, another type III IF protein, involve a set of sequences similar to those important for desmin binding; 5) the potential of DP and plectin to interact with desmin and vimentin in yeast appears to be differently affected by phosphorylation of Ser 2849 and Ser 4532, respectively, within their COOH termini.

Together, these results demonstrate that DP and PL can directly interact with desmin and that the linker region and COOH extremity of DP and PL contain recognition sites critical for desmin binding. These observations provide novel insights into the cytoskeletal organization of striated muscle cells and increase our understanding of plakin-related human diseases associated with both skin and muscular defects.

003 [Oral 050]

Mapping of BP230, Desmoplakin and Plectin Binding Sites on K5/K14 Heterodimer by Yeast Three-Hybrid Assays and Impact of Keratin Mutations on their Association with Plakins

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The bullous pemphigoid antigen 1 (BP230), desmoplakin (DP) and plectin (PL) are members of the plakin protein family of cytolinkers. Despite their homology, their COOH termini selectively bind to distinct intermediate filaments (IF). Previously, we have provided evidence indicating that the binding specificity of plakins for various IF proteins depend on their linker region between the highly homologous B and C subdomains and their COOH extremity. Furthermore, the association of DP and BP230 with both epidermal and simple keratins is critically affected by the tertiary structure induced by heterodimerization, and involves recognition sites primarily located in the rod domain of these keratins. We have here started to map the binding sites for BP230, DP or PL on keratins and analyzed the impact of pathogenic mutations of K5 and K14 on their ability to heterodimerize and to associate with BP230, DP and PL in yeast three-hybrid assays. The results indicate that: 1) the NH2-terminal half of K5 and K14 are sufficient to mediate binding to BP230, DP and PL whereas the head and tail domain of K5 and K14 do not bind to BP230, DP and PL. We show that the K5 mutations E168D and E168Q both located in the H1 subdomain of the K5 head and the N177S mutation located in the 1A segment of the rod domain apparently increase the ability of K5 to dimerise with K14 whereas the N176S mutation have no effect. Furthermore we found that the K14 mutations R125H and L284P located respectively in the 1A and 2A segment of the K14 rod respectively, had only minor impact on the ability of K14 to form homodimers with K5, whereas the K14 V270M mutation located in the L12 segment of the K14 rod strongly affected the binding of K14 to K5; finally, 4) the substitution E168Q or N176S in K5 increased the interaction of K 5/K14 with DP, whereas the K14R125H mutant showed reduced binding to BP230. In conclusion, we have found that the NH2-terminal half of the rod domain of K5 and K14 contains the binding sites for three different members of the plakin family of protein, BP230, DP and PL. Our findings obtained with different K5 or K14 mutants suggest that these mutations not only affect the formation of K5/K14 heterodimers and hence the assembly of keratin filaments but also interfer with the connection of IF to the plasma membrane by altering their potential to associate with cytoskeletal linkers such as BP230, DP and PL. These findings provide novel insights into the molecular mechanisms responsible for the disturbed cyto-architecture and cell fragility associated with epidermolysis bullosa simplex.

004 [Oral 051]

The Distorted Keratin Filaments are Transported and Re-Organized Using the Cytoplasmic Dynein Cargos

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Epidermolysis bullosa simplex with mottled pigmentation (EBS -MP) is a rare variant of EBS and caused by a specific missense mutation located at the early head domain of keratin 5 (K5). The mechanism, which leads to the blister formation as well as pigment anomaly in EBS-MP, is not yet understood at all. Last year we reported that a tctex1, a cytoplasmic dynein light chain (DLC), bind to the critical region of K5 by yeast two-hybrid system. The interaction of DLC with K5 was further confirmed by pull down as well as co-immuno-precipitation assays. However, dynein complex did not consistently co-localize with keratin filaments in the immuno-cytochemically observed non-treated keratinocytes. We then tried to observe and determine the interaction of keratin, tubulin and dynein complex each other after various stimuli on cultured keratinocytes. Various kinase or phosphatase inhibitor treatments immediately induced the marked breakdown of keratin filaments to the granular deposition. After washing out these chemicals, keratins were observed to gradually recover their filamentous formation. During the recovery process, keratins and dynein complex were found to well co-localize along the microtubule. The nocodazole treatment prior to the kinase treatments completely disturbed this re-organization step of keratin filaments. Our observation above suggests that the dynein system is necessary for the dynamic maintenance of healthy keratin networks, and the disturbance of this recovery process must be the cause of blisters in EBS-MP patients.

005 [Oral 052]

Derivation and Isolation of Ectodermal and Epidermal Progenitor Cells from Murine Embryonic Stem Cells

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Presence of multipotent stem cells allow physiological renewal of the cutaneous epithelium and skin appendages (hair follicles, sweat and sebaceous glands). However the biopsy-derived cultured keratinocytes, which are used for decades to treat deep extensive burn wounds and chronic ulcers, do not form skin appendages in grafted epithelium. During embryogenesis, ectodermal cells (the precursors of multipotent keratinocyte lineages) switch their keratin production from K8/18 to K5/14. This embryonic transition is abolished in p63 knockout mice but rescued by exogenous expression of TAp63. We recently found culture conditions to efficiently differentiate embryonic stem (ES) cells into keratinocytes that were able to reconstitute *in vitro* a fully differentiated epidermis. The aim of our work was to isolate both ectodermal K8/18 and epidermal K5/14 progenitor cell lines, from ES cells, that would retain epidermal multipotency *in vitro*. ES cells committed to keratinocyte differentiation recapitulate the *in vivo* embryonic epidermal fate since large amount of K8/K18 positive cells were detected early in differentiation before appearance of K5/K14 keratinocytes. By serial passages at clonal density, an ES-derived K8/18 cell line was isolated. The cell line proliferates at normal rate, retains typical epithelial morphology and remains stable for its keratin content. Gene expression profil analysis strongly suggests this cell line to be characteristic of epidermal lineage and not to simple epithelia. Interestingly, exogenous expression of TAp63 isoform, and not ΔNp63, was able to convert ES-derived K8/K18 cells into K5/K14-expressing cells, demonstrating its ability to differentiate into early keratinocyte stem cells. Grafting experiments are currently conducted to demonstrate the multipotency of this cell line *in vivo*. The applications of this cellular model are to identify molecules essential for the multipotent epidermal fates and to characterize the target genes of TAp63 during these commitments.

006 [Oral 048]

The Cystatin M/E – Legumain Balance: a Regulator of Epidermal Cornification

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Cystatin M/E is a recently discovered cysteine protease inhibitor that is highly expressed in cutaneous epithelia. We have previously found that homozygosity for null alleles of the cystatin M/E gene causes the phenotype of the *ichq* mouse, which resembles a severe form of human ichthyosis. Here we have studied the mechanism by which deficiency of cystatin M/E leads to disturbed cornification, using *in vivo* and *in vitro* models. Absence of cystatin M/E causes unrestricted activity of its target protease legumain in hair follicles and epidermis, which is the exact location where cystatin M/E is normally expressed. Biochemical analysis of stratum corneum proteins revealed a strong decrease of soluble loricrin monomers in skin extracts of *ichq* mice, although loricrin was normally present in the stratum granulosum and stratum corneum of *ichq* mice, as determined by immunohistochemistry. This suggested a premature or enhanced crosslinking of loricrin monomers in *ichq* mice by transglutaminase 3 (TGase-3). In these mice we indeed found strongly increased levels of TGase-3 that was processed into its activated 30 kDa and 47 kDa subunits, compared to wild type mice. Legumain was found to mediate the activation of TGase-3 zymogen via lysosomal cysteine proteases *in vitro*. This study shows that cystatin M/E and legumain form a functional dyad in epidermis *in vivo*. Disturbance of this protease-antiprotease balance causes increased enzyme activity of TGase-3 that could explain the observed abnormal cornification.

007

The 27kD Heat Shock Protein and MAPK Signalling Regulate the Expression of Differentiation-Associated Proteins in Human Keratinocytes

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In human epidermal keratinocytes the expression of hsp27 is closely related to differentiation *in vitro* and *in situ*. Hsp27 is a molecular chaperone and its protein binding activity is associated with phosphorylation/dephosphorylation that is catalyzed through MAP-kinase signalling. This project was aimed to gain further insight into the role of hsp27 in keratinization by overexpression, co-localization studies, and inhibition of the key enzyme in hsp27 phosphorylation, p38/MAPK.

Co-localization of hsp27 with loricrin, filaggrin, transglutaminase1, actin, and keratins was investigated by confocal laser scan microscopy in calcium-differentiated normal human keratinocytes (NHEK), in "living skin equivalents", and in the squamous cell carcinoma cell line A431 transfected with the human hsp27 gene. Protein expression was investigated by Western blot. SB203580, a specific inhibitor of p38-MAPK, was used to investigate the role of hsp27 phosphorylation in keratinocyte differentiation.

In accordance with its role as a molecular chaperone we found co-localization of hsp27 with differentiation antigens in A431, NHEK, and the epidermis of artificial skin. Artificial skin behaved close to normal human epidermis regarding co-localization of hsp27 with other proteins. Both, overexpression of hsp27 in A431 and inhibition of the hsp27-phosphorylation by SB203580 in NHEK changed the expression of differentiation associated proteins. A further novel result of this study is that inhibition p38-MAPK not only inhibits phosphorylation but also leads to decreased expression of hsp27 in NHEK. These results provide evidence that hsp27, probably through its function as a chaperone, is involved in the regulation of the coordinated expression of differentiation associated proteins in keratinocytes. Furthermore, hsp27 might provide a molecular link between MAPK activation and keratinocyte differentiation. Pharmacologic inhibition of hsp27 phosphorylation and expression as described here will not only help to further investigate the differentiation process but also provides a new potential target for therapeutic intervention.

008

Drebrin, an Actin-Binding Protein of Diagnostic Potential in Dermatology: Induction and Localization in Epithelial Skin Tumors and Cultured Keratinocytes

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Drebrins are actin-binding proteins originally identified in neuronal cells and thought to be involved in the formation of neuronal cell processes. More recently, we have detected that the isoform drebrin E2 also occurs in many different non-neuronal cell types where it is enriched both in cell processes and at intercellular junctions. Here we report on the distribution of drebrin in normal and pathologically altered human skin as well as in keratinocytes in cell culture. In normal human skin the keratinocytes contain almost no drebrin; however, this protein is enriched in the companion layer and outer root sheath of hair follicles. Interestingly, the intercellular junctions of basal cell carcinomas are extremely rich in drebrin, as shown by immunohistochemistry and immunoblot analysis. Using confocal laser scanning microscopy, we have observed colocalization of drebrin with actin and partially also with plaque proteins of adhering junctions. In squamous cell carcinomas and precancerous lesions the distribution of drebrin can be rather heterogeneous, with drebrin-rich and drebrin-negative tumor regions. Under cell culture conditions, primary human keratinocytes synthesize significant amounts of drebrin, accumulating again along adhering junctions. When cultured epithelial cells containing no endogenous drebrin, i.e. A-431 human vulvar carcinoma cells, have been transiently or stably transfected with constructs of drebrin cDNA fused with EGFP, accumulation of the EGFP-tagged drebrin along the adhering junctions has been observed. Moreover, immunoprecipitation experiments have shown biochemical interactions of drebrin with certain plaque proteins of adhering junctions. Our results indicate an involvement of drebrin in regulating intercellular adhesions of somehow "activated" keratinocytes. The strong upregulation of this protein in basal cell carcinomas, also arguing for their follicular origin, points to the possibility of diagnostic application.

009

Retinaldehyde-Induced Epidermal Hyperplasia via Heparin Binding Epidermal Growth Factor is CD44-Dependent

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CD44 is a multifunctional polymorphic proteoglycan involved in broad cellular processes. CD44 functions as the principal cell-surface receptor for hyaluronate (HA). Retinoids are known to inhibit keratinocyte differentiation and to stimulate epidermal hyperplasia. Heparin binding epidermal growth factor (HB-EGF) activation of keratinocyte ErbB receptors has been proposed to mediate retinoid-induced epidermal hyperplasia in human skin organ cultures. It has been shown that a heparan sulfate-bearing variant of CD44 (CD44v3) recruits proteolytically active matrix metalloproteinase 7 (MMP-7), the precursor of HB-EGF (pro-HB-EGF) and one of its receptors, ErbB4 to form a complex on the surface of some tumor cell lines, uterine and mammary gland epithelium, and uterine smooth muscle. In this study we first analyzed the effect of a retinoid, retinaldehyde (RAL), on *in vitro* proliferation of keratinocytes from SKH1 hairless, DBA/1 and CD44-deficient ($CD44^{-/-}$) mice. Treatment of primary keratinocyte cultures of SKH1 hairless and DBA/1 mice with RAL resulted in slight but significant increase in keratinocyte proliferation whereas no proliferation was observed in $CD44^{-/-}$ cells. RAL also induced *in vitro* HA production by keratinocytes. While HB-EGF stimulated normal and CD44-deficient keratinocytes to proliferate, blocking antibodies against HB-EGF and erbB1, and tissue inhibitor of metalloproteinase-3 (TIMP-3) which inhibits the effect of metalloproteinases including MMP-7, abrogated the RAL-induced keratinocyte proliferation. We then analyzed the effect of RAL on epidermis in SKH1 hairless, DBA1 and $CD44^{-/-}$ mice. Topical application of 0.05% RAL for 3 days resulted in a significant epidermal hyperplasia and keratinocyte proliferation as determined by quantitation of Ki67 in the back skin of SKH1 hairless and DBA/1 mice, whereas no epidermal hyperplasia and keratinocyte proliferation was observed in $CD44^{-/-}$ mice. Topical application of RAL also significantly induced the CD44 protein expression in the follicular and interfollicular epidermis and increased the epidermal HA in SKH1 hairless and DBA/1 mice, as determined by immunohistochemistry and ELISA, respectively. Western blot analysis of RAL-treated epidermis showed an increase of CD44v3, active HB-EGF and erbB1 protein expression when compared to vehicle-treated epidermis in SKH1 hairless mice. Our results indicate that RAL-induced *in vitro* and *in vivo* proliferative response of keratinocytes is a CD44-dependent phenomenon and requires the presence of HB-EGF, erbB1 and matrix metalloproteinases.

010 [Oral 066]

CD44-Dependent Mouse Skin Hyperplasia Induced by Topical Hyaluronate Fragments

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Hyaluronate (HA) is a major glycosaminoglycan component of the extracellular matrix, and present mainly in dermis and also in epidermis. In its native form HA exists as a high molecular weight polymer but is cleaved to lower molecular weight fragments during inflammatory processes. High molecular weight HA is capable of immobilizing the water in the tissue and therefore of changing the dermal volume and elasticity to maintain the extracellular space. Several studies have suggested that high and low molecular weight HA may exhibit different biological effects on cells and in tissues. Many of the functions of HA are thought to be mediated by its cell surface receptor, CD44. CD44 is a polymorphic transmembrane glycoprotein and was shown to form a complex with matrix metalloproteinase 7 (MMP-7), heparin binding epidermal growth factor (HB-EGF) precursor (pro-HB-EGF) and one of its receptors in mouse uterine and mammary epithelia. In this study we first examined the effect of HA fragments (HAF) of different size that we generated from high molecular weight HA by sonification, enzymatic digestion and size exclusion gel filtration, on *in vitro* proliferation of keratinocytes from SKH1 hairless, DBA/1 and CD44-deficient ($CD44^{-/-}$) mice. Treatment of primary keratinocyte cultures of SKH1 hairless and DBA/1 mice with HAF of intermediate (HAFi; 50,000-400,000 Da) but not of small (HAFs; 1,000-50,000 Da) or large (HAFl; 400,000-1,000,000 Da) size resulted in a significant increase in keratinocyte proliferation whereas no proliferation was observed in $CD44^{-/-}$ cells. HB-EGF stimulated the proliferation of normal and CD44-deficient keratinocytes whereas blocking antibodies against HB-EGF and its receptor, erbB1, and tissue inhibitor of metalloproteinase-3 (TIMP-3) which inhibits the effect of metalloproteinases including MMP-7, abrogated the HAFi-induced keratinocyte proliferation. We then examined the effect of HAFi on epidermis in SKH1 hairless, DBA/1 and $CD44^{-/-}$ mice. Topical daily application of 0.2% HAFi but not of HAFs or HAFl for 3 days to the back skin of SKH1 hairless and DBA/1 mice resulted in a marked epidermal hyperplasia and keratinocyte proliferation as determined by quantitation of Ki67, and in structural dermal alterations and an increase in the number of dermal cells, mainly of fibroblasts visualized by electron microscopy. Topical application of HAFi also significantly induced the CD44 protein expression in the follicular and interfollicular epidermis as determined by immunohistochemistry. Topical HAFi also increased the HA content and induced the mRNA expression of hyaluronate synthase-2 (HAS2) and HAS3 in the epidermis and dermis of SKH1 hairless and DBA/1 mice, as evidenced by ELISA and Northern blot analysis, respectively. In contrast, topical application of HAFi to the back skin of CD44-deficient ($CD44^{-/-}$) mice did not reveal any epidermal hyperplasia, keratinocyte proliferation or dermal alterations. Western blot analysis of HAFi-treated epidermis showed an increase of CD44, pro- and active HB-EGF, erbB1 and MMP-7 protein expression when compared to vehicle-treated epidermis in SKH1 hairless mice. These results suggest that HAFi-induced *in vitro* and *in vivo* proliferative response of keratinocytes is mediated via a CD44-dependent pathway and requires the presence of HB-EGF, erbB1 and matrix metalloproteinases.

011

Protein Kinase C Isoforms have Different Effect on the Expression of Desmoglein 1, 3 and P-cadherin in HaCaT Keratinocytes

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Protein kinase C (PKC) comprises a family of serine/threonine kinases and emerging evidence suggests a privotal role for these enzymes in the regulation of the proliferation and differentiation of normal human epidermal keratinocytes. In addition, isoenzyme-specific roles of PKC in keratinocyte proliferation and differentiation have been described. Our laboratory has previously published the construction of stable PKC transfectants of HaCaT cells and has presented clear evidence for the specific and antagonistic roles of certain conventional (cPKC) and novel (nPKC) PKC isoforms in the regulation of proliferation and differentiation of the human keratinocyte line HaCaT.

Epithelial cells utilize multiple adhesive mechanisms to maintain the structural integrity of tissues, including adherens junctions and desmosomes. Adherens junctions consist of classical cadherins like P-cadherin and desmosomes consist of desmosomal cadherins, desmogleins (Dsg) and desmocollins. These molecules are expressed in a differentiation-specific fashion in the epidermis, with Dsg3 and P-cadherin being present in the basal and spinous layer and Dsg1 predominanly in the granular layer.

Our objective was to investigate the effect of overexpression of PKC isoforms on the production of Dsg1, 3 and P-cadherin in HaCaT cells. We used Western blot analysis.

Our results indicate that the overexpression of PKC isoforms altered the expression of desmosomal and classical cadherins. Overexpression of cPKCα and nPKCδ increased the expression of Dsg1 and decreased the production of Dsg3 and P-cadherin compared to overexpression of cPKCβ and nPKCε in HaCaT cells. Incubation of HaCaT cells with the nPKCδ inhibitor rottlerin, resulted in the stimulated production of Dsg3 and P-cadherin.

These finding are in good correlation with our previous results as PKC isoenzymes have isoform specific roles in the regulation of various keratinocyte functions; cPKCα and nPKCδ stimulated cellular differentiation and in contrast cPKCβ and nPKCε increased growth of cells.

012

Coordination Between Epidermal Cell Growth and Differentiation: Novel Mechanisms

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Human epidermis is a frequent target of hyperproliferative disease. However, the mechanisms that link cell growth and differentiation and maintain epidermal homeostasis are not understood. We have previously shown that primary differentiating keratinocytes continue DNA synthesis and become polyploid in the absence of mitosis (endoreplication). Cell growth, cell cycle, differentiation and genomic stability are thus tightly linked. We have unravelled this phenomenon not only in primary cultured keratinocytes but also in human epidermis *in situ*. To this aim, we have applied to human skin up-to-date techniques for the study of cell cycle, DNA replication and genome amplification. This phenomenon is regulated and stimulated by the proto-oncogene c-myc, and we are investigating the molecular regulation. The results show a particular combination of cyclin/cdks, Rb, p53 and the p21 inhibitor that hallmarks a mitosis block and endoreplication in the onset of differentiation. Finally, we have demonstrated the physiological importance of this control in epidermal-specific c-myc knock-out mice. This novel phenomenon may be central in co-ordinating proliferation with differentiation and in protecting epidermis against hyperproliferative alterations.

013

Migration Characteristics of laminin 5 Deficient Keratinocytes

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Laminin 5 is an important extracellular matrix component involved in keratinocyte migration, e.g. during epidermal wound healing. To determine the significance of laminin 5 for cell migration in detail we quantified the main parameters of migration such as lamellipodia protrusion and persistence, ruffle frequency as well as cell translocation velocity using the stroboscopic analysis of cell dynamics assay. In laminin 5 deficient cells on fibronectin we found a significantly (40-50%) reduced velocity of lamellipodia protrusion and persistence, whereas the frequency of ruffle formation was about 3-fold higher than in wild type cells. In spite of these findings, we observed no differences in the translocation velocity between deficient and wild type cells. However, on glass the deficient cells showed normal dynamics of lamellipodia and ruffles but a significantly decreased translocation velocity. To further characterize the migration and adhesion process in laminin 5 deficient keratinocytes we analyzed the formation of migration tracks at the rear of migrating cells. Keratinocyte migration tracks consist of extracellular matrix components and integrin macroaggregates, i.e. membrane bounded fragments that are enriched in different integrins and that result from membrane ripping at the cell rear during the process of rear release. The ultrastructural analysis of the migration track of laminin 5 deficient keratinocytes revealed a lack of spherical and elongated tubular structures, that are thought to contain alpha6 beta4 integrin and are regularly found in tracks of wild type cells. In spite of significant differences in the mechanisms of cell migration laminin 5 deficient keratinocytes are capable of efficient cell migration when fibronectin is used as substrate which obviously can compensate for the lack of laminin 5.

014

Cytokines Induce Neuropeptide Galanin Expression in Human Keratinocytes

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Galanin (GAL), a 29 amino acid neuropeptide, has multiple effects in both the central and peripheral nervous system. In the human skin GAL is mainly detected in the epidermis and in sweat glands in a non-neuronal distribution (*Kofler et al J Invest Dermatol 2004, in press*). RT-PCR and northern blot analysis of human primary cultured epidermal foreskin keratinocytes (KC) revealed detectable levels of GAL mRNA. In order to uncover a possible function of GAL in the human epidermis we investigated the regulation of GAL gene expression in KC. The known regulators of GAL mRNA expression PMA (50 nM) and leukemia inhibitory factor (LIF, 10 ng/ml) induced GAL mRNA in KC 6.1-fold and 4.5-fold, respectively. Upon stimulation of KC with a combination of IL-1β (10 ng/ml) and TNFα (100 ng/ml) for 24 hrs a 2.2-fold induction of GAL mRNA was detected. In addition, a 5-fold induction upon treatment with 100 ng/ml IL-4 for 6 hrs was observed. Treatment of KC with Il-6, histamine, UV or heat did not alter GAL mRNA levels. Interestingly, treatment of KC with the VR1 agonist capsiacine (1 μM) leads to a 2-fold down regulation of GAL mRNA. In contrast treatment of KC with the Vanilloid receptor 1 antagonist capsazepine (10 μM) resulted in a 3-fold up-regulation of GAL mRNA. The regulation of galanin mRNA expression in human keratinocytes by cytokines and neuro-modulatory factors suggests a role of GAL in inflammatory processes in the human skin.

015

Expression of D-type Cyclins in HaCaT Keratinocytes

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Amongst the cell cycle regulator cyclins, only D-types are known to have different isotypes. A strong α5 integrin expression appears in HaCaT keratinocytes when the cells leave the Go, quiescent phase, and enter the cell cycle, indicating that α5 integrin regulates keratinocyte proliferation. We were interested to see, whether in HaCaT keratinocytes, similar to normal keratinocytes, D-type cyclins are expressed differentially in Go-G_1/S and G_1/S phases of the cell cycle, and whether α5 integrin regulates the expression of D-type cyclins. We have forced HaCaT cells into quiescent (Go) phase by serum withdrawal and high density culturing, then released them into an almost synchronized highly proliferative state by passaging into serum containing medium. The expression of D-type cyclins was determined with Real-Time RT-PCR. The regulatory role of α5 integrin was examined by adding neutralizing anti-α5 integrin monoclonal antibodies to the HaCaT cell culture. Our results showed that HaCaT keratinocytes expressed all three D-type cyclins (D1, D2 and D3). The Go-G_1/S phase of the cell cycle can be characterized by the appearance of D1 cyclin, while during the rapid turnover in highly proliferating cells (G_1/S transit) the expressions of cyclins D2 and D3 are much more significant. Blocking the function of α5 integrin before the cells leave cell quiescence results in a significant decrease in D1 cyclin expression, indicating that α5 integrin takes part in regulating the cell cycle at this point.

016

Peptidylarginine Deiminases: Genomic Characterization and Expression Analysis in the Epidermis

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Peptidylarginine deiminases (PADs) convert arginine residues in proteins into citrullines. They are suspected to be involved in multiple sclerosis and rheumatoid arthritis pathophysiology, and they play a pivotal role in epidermis homeostasis. In humans, four isoforms encoded by the genes *PADI*1-4 were known so far. Their substrate specificity and expression pattern in the epidermis are still unclear. We characterized the human (355 kb) and mouse (240 kb) *PADI* gene clusters on chromosomes 1p35-36 and 4E1, respectively. A yet unknown human gene, *PADI*6, was identified which is expressed in ovary, testis and peripheral blood leukocytes, and encodes a 694-amino-acid protein. The genes *PADI*1-6 may have duplicated from a common ancestor and appeared to be under purifying selection, as suggested by their nucleotide substitution rates (dN/dS). To gain more information on the physiological role of PADs in the epidermis, we investigated their expression pattern. mRNA analysis, western blotting and confocal laser microscopy analyses using anti-peptide antibodies highly specific to each PAD isoform revealed that only PAD1-3 are expressed in the human epidermis. PAD1 was detected in the entire epidermis, including the stratum corneum (SC). PAD2 was found in all the living layers, and accumulated at the keratinocyte periphery in the granular layer. PAD3 expression was found to be restricted to the granular layer and lower SC. PAD1 and PAD3 co-localized with (pro)filaggrin. Such different patterns of expression indicated a fine regulation of the *PADI* gene expression, and prompted us to search for possible regulators of *PADI* gene expression at the genomic level. Comparative analysis of the *PADI* gene clusters identified 251 highly conserved non-coding segments predominantly clustered within the promoter regions, the large (>10 kb) first intron of each of the genes *PADI*1-3, and an 8 kb *PADI*1-2 intergenic region. The presence of numerous transcription factor binding sites suggests these genomic regions are putative regulatory elements. Our results strongly suggest that each epidermal PAD has a specific role in epidermis differentiation, and will help unravelling the genomic bases of their co-ordinated expression in the skin. Also, this study is the first step towards the substrate specificity of epidermal PADs.

017

Differentiation of Murine Embryonic Stem Cells into Keratinocyte-Like Cells, their Isolation and Cultivation

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Embryonic stem (ES) cells are omnipotent; they can differentiate into every cell type of the body. The signals that regulate differentiation into squamous epithelial cells are poorly defined. *In vitro* production of large numbers of epidermal cells from embryonic stem cells could be used in dermatology in order to cover large wounds, e.g. of burn victims. Elimination of immunogenic determinants in ES cells prior to differentiation would eventually allow to generate non-immunogenic epidermal cells that could be universally used for different recipients. In order to understand the signals that regulate differentiation of ES cells into squamous epithelial cells we have used embryoid bodies (EBs) formed from embryonic stem cells as a system for *in vitro* differentiation. EBs were first cultured in suspension and then plated onto tissue culture dishes. At day 17 of culture cells formed colonies of epithelial cells within sheets of non-epithelial cells. These colonies expressed epithelial cadherin (E-cadherin), a classical type I cadherin and a key regulator of epithelial cell-cell adhesion in embryonic and adult tissues. During subsequent days a subset within the epithelial layer started to express keratin 14, a type I keratin, which is constitutively present in basal keratinocytes of the epidermis and stratified epithelia and in the outer root sheath of the hair follicle. Many of these cells were larger than the surrounding cells and showed filamentous keratin structures. Keratin 14 positive cells subsequently merged to form aggregates. We were able to isolate such cells from the EBs and culture them *in vitro* for several days. Our results show that embryonic stem cells that form an EB are able to differentiate into keratinocyte like cells and that these cells can be isolated and cultured *in vitro*.

018

Caspase-14 Derived From Stratum Corneum of Human Epidermis is Catalytically Active

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Caspase-14 is expressed by epidermal keratinocytes as a proenzyme which undergoes processing to a large and a small subunit during terminal keratinocyte differentiation. Here we demonstrate for the first time that caspase-14 present in human stratum corneum is catalytically active. Stratum corneum was scraped off from plantar skin and extracted with phosphate buffered saline. Western blotting revealed presence of caspase-14 subunits whereas procaspase-14 was absent both in the soluble and the insoluble fraction. By contrast, extracts from parakeratotic epidermis contained both cleavage products and procaspase-14 indicative of incomplete proenzyme activation. Under kosmotropic salt conditions, stratum corneum extracts displayed significant cleavage activity against the synthetic tetrapeptide substrates WEHD-pNA and IETD-pNA as measured in a colorimetric assay. This activity correlated with the concentration of processed caspase-14 and could be specifically removed by immunodepletion of caspase-14. Our results indicate that, in normal epidermis but not in parakeratotic epidermis, practically all procaspase-14 is converted to caspase-14 subunits which have the potential to act proteolytically. The identification of tetrapeptide sequences recognized by caspase-14 is likely to facilitate the identification of the physiological substrate of this epidermis-specific enzyme.

019

Expression of Skin Specific DNase1L2 is Linked to Terminal Differentiation of Keratinocytes

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Degradation of nuclear DNA is a hallmark of terminal differentiation of keratinocytes. As the enzymes mediating this process are unknown at present, we performed real-time PCR screening of candidate DNases. While most DNase genes were expressed at comparable rates in pre- and postconfluent keratinocytes, DNase1L2 was strongly upregulated during keratinocyte differentiation. Moreover, the DNase1L2 mRNA level was found to be approximately 100-fold higher in skin than in all other tissues examined. In skin, DNase1L2 mRNA abundance increased significantly from the basal layer towards the granular layer of the epidermis as demonstrated by *in situ*-hybridization. Recombinant expression of DNase1L2 in Escherichia coli yielded inclusion bodies whereas expression in the yeast Pichia pastoris allowed for production of a secreted enzyme that was able to degrade plasmid DNA *in vitro*. Both protein preparations were used for the immunization of rabbits. The polyclonal antibodies detected a specific band in Western blots of protein lysates from human skin equivalents and revealed a gradient of increasing expression in the epidermis. The keratinocyte-specific and differentiation-associated expression of DNase1L2 suggests that this enzyme participates in DNA degradation during terminal differentiation of keratinocytes.

020

Decreased Peroxisome Proliferator-Activated Receptor MRNA Expression in Cultured Keratinocytes after UVB Irradiation

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate the expression of target genes involved in cell differentiation, proliferation, apoptosis, metabolic processes and immune response. The PPAR family consists of PPAR alpha, PPAR delta and PPAR gamma isotypes all of which are expressed in keratinocytes. According to previous studies PPAR delta is the predominant subtype in human keratinocytes, whereas PPAR alpha and PPAR gamma are expressed at lower levels but show significant increase upon keratinocyte differentiation. PPAR delta is upregulated during keratinocyte proliferation. UVB light induces keratinocyte apoptosis, inflammatory reaction and latter on keratinocyte proliferation *in vivo*. Few studies have been carried out to understand the effects of PPARs during UVB irradiation. We used an *in vitro* system: immortalized human keratinocytes (HaCaT cells) to study the expression of PPAR mRNAs after UVB irradiation. mRNA expression was studied by quantitative RT-PCR reaction and normalized to cyclophilin mRNA levels. Broad band UVB light source (FS20 tubes) was used for 30-120 mJ/cm^2 irradiation. Time course and dose dependence were determined. The level of DNA damage of irradiated keratinocytes was measured by comet assay. Cell viability was also determined and correlated with PPAR mRNA levels. Apoptotic and proliferative effects of UVB were measured by flow cytometry. After UVB irradiation the expression of all PPAR mRNAs was significantly decreased. Our results suggest that PPARs may play a role in the regulation of the effects of UVB irradiation in human keratinocytes.

021

Proteoglycans in the Intercellular Cores of Desmosomes and Corneodesmosomes

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Proteoglycans (PG) are complex glycoproteins composed of a protein core with specifically attached sugar chains of glycosaminoglycans (GAG). Various forms of PG are present in many different tissues, including human epidermis. We have previously defined desmosealin (recognized by KM48 antibody) as an epidermal PG which differs from the others, mostly of the heparan sulfate (HS) type, by its selective chondroitin / dermatan sulfate (CS/DS) GAG expression. Desmosealin is also a constitutive desmosomal antigen showing an expression pattern positively correlated with the keratinocyte differentiation. However, it disappears rapidly in the lower portions of the horny layer. Another monoclonal antibody, 7C1, obtained after immunization of mice with a whole desmosome fraction isolated from a normal human epidermis, shows an expression profile very similar to KM48. Ultrastructural immunolocalization on ultrathin cryosections of a normal human skin reveals the presence of both antigens in the inter-keratinocyte spaces and their integration into the desmosomes. Biochemical analysis demonstrates that 7C1 antigen also belongs to the PG family. However, its GAG are not only of the CS/DS but also of the HS type. Cross-immunoprecipitation assays with antibodies to syndecan-1, CD44/HCAM, KM48 and 7C1 do not reveal any cross-reactivity, thus indicating that desmosealin and 7C1 antigen are different from the known epidermal PG displaying a similar, pericellular distribution.

Although KM48 antibody proves difficult to use in immunoprecipitation assays, the surface plasmon resonance (BIAcore®) technology can be employed for 'ligand fishing' and opens up the possibilities of subsequent mass spectrometry analysis of the studied antigens.

Provided the influence of PG on such fundamental biological events as cell recognition, proliferation, and differentiation, and the evidence of the protective role of sugars against the proteolytic degradation of desmosomes and corneodesmosomes, we speculate that the desmosome-associated PG may be involved in the regulation of epidermal cohesion and desquamation.

022

Oxidative Stress-Induced Cell Death in Human Epidermal Keratinocytes is Dependent on their Differentiation State

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Epidermis, is a multilayered structure composed of basal proliferating and suprabasal growth arrested keratinocytes that differentiate into corneocytes. Due to their external position, keratinocytes are particularly exposed to oxidative stresses among which H_2O_2 plays a pivotal role. Apoptosis is a physiologic programmed cell death that occurs throughout the epidermis contributing to epidermal protection and maintenance by removing damaged or unwanted cells. Recent evidences suggest that keratinocytes susceptibly to apoptosis is dependant on their differentiation state. The present study investigates the response of basal and suprabasal human keratinocytes to a calibrated H_2O_2-induced cell death. On H_2O_2-exposed skin explants, caspase-3, a key effector in apoptosis, was strongly activated in basal keratinocytes double stained with β1-integrin antibody, since apoptotic single strand DNA cleavage was restricted to the granular layer, the spinous layer being unresponsive. In addition, using cytometric analysis, isolated basal human keratinocytes, selected on adhesion criteria to type IV collagen, were more sensitive than non-adherent cells to H_2O_2-induced apoptosis regarding mitochondrial transmembrane potential collapse and plasma membrane integrity, respectively assessed with JC-1 and TOTO-3 probes. In the same way, necrotic/late apoptotic cells were present at low levels only in adherent epidermal population. We concluded that basal proliferating undifferentiated keratinocytes are much more sensitive than suprabasal growth arrested differentiated ones to oxidative stress-induced cell death and that apoptosis in human keratinocytes could occur via different pathways depending on their differentiation state.

023

Apoptosis and Proliferation During Human Epidermal Aging after an Acute UV Exposure: an *In Vivo* Immunohistological Study

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Ultraviolet radiations (UVR) participate to skin carcinogenesis and photoaging. When epidermis, the outermost part of the skin, is exposed to UVR, damaged keratinocytes can undergo apoptosis, a programmed cell death that removes them from the tissue contributing to its protection and maintenance. Therefore the question related to a decrease of such a phenomenon in human skin during aging raises. The aim of this study was to investigate, depending on the age, apoptosis in normal skin following an acute solar simulated radiation (SSR).

Apoptosis was assessed in the sun-exposed skin of the forearm of young (n = 12, mean age = 25.5) and aged female volunteers (n = 12, mean age = 67.1) before and 24h after a single SSR to a 3-fold minimal erythema dose (MED). Immunohistological studies were carried out on 10 μm cryosections obtained from 3 mm skin biopsies. Apoptosis and proliferation were investigated using antibodies against active caspase-3 and Ki-67 respectively.

The results indicate that the proliferation index (PI) is about two times greater in the young than in the aged epidermis. However, 24 h after SSR exposure, while proliferation was greatly inhibited in the young epidermis (PI from 216 to 68), it slightly decreased in the aged (PI from 115 to 85). Apoptosis was strongly induced by UVR in both groups with a greater sensitivity of the young volunteers as opposed to the aged ones. For all volunteers, no spontaneous apoptosis was observed before SSR exposure. In addition, double staining with β_1 integrin, a marker of basal epidermal keratinocytes, indicated a percentage of active caspase-3 positive cells in the basal compartment greater in the aged volunteers than in the younger. Taken together, these results indicate that an acute SSR exposure to 3 MED induced apoptosis more strongly in young than in aged epidermis from chronically photo-exposed areas, and that this greater sensitivity of young epidermis is associated with an arrest of basal cell proliferation.

024

Human Endogenous Retroviruses in Normal Human Skin

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A part of the Human genome consists of repeated sequences of unknown functions. Among them, Human endogenous retroviruses represents up to 7%. They are reminiscent of ancestral retroviral infection that lost their infectiosity through acquisition of point mutations or deletions. However, these sequences still contain active promotor and ORFs that could lead to the production of viral proteins. In the present work, we first evaluated which HERV family was expressed in normal skin, detected viral proteins and tested their expression under UV irradiation.

By using the pan-RetroPCR based on conserved sequence among reverse transcriptase, we observed in normal human skin that most of the HERV transcripts belonged to the MLV-like superfamily including HERV-W and HERV-E, the remaining transcripts belonging to the MMTV superfamily, namely HERV-K. Interestingly, when we tested for a Mn2+ dependant reverse transcriptase in normal human skin, we found a weak activity (mean = 61 μU/ml, n = 10), above the cut-off of significativity.

At the protein level, numerous capside MLV-like proteins are detected with a predominant protein at 55 kD. Also, HERV-E envelope protein is detected with a M.W. of 57 kD. Both proteins were also detected in cultured keratinocytes. The expression of these proteins was strongly down-regulated by UVB irradiations (30mJ/cm^2).

These types of sequences were, so far, considered as non-conventional genes or "junk DNA". These results suggested that they could be expressed, UV-regulated and functional for at least some of them. Their role in skin physiology remains to be investigated.

025

Scavenger Receptors Expression at the Cutaneous Level

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Scavenger receptors (SRs) are a family of cell surface glycoproteins; they are expressed by myeloid cells (macrophages and dendritic cells) and certain endothelial cells. They play an important role in uptake and clearance of effete components, such as modified host molecules and apoptotic cells. They are able to bind modified lipoproteins such as oxidised and acetylated LDLs (oxLDL and acLDL). They bind and internalise microorganisms and their products. Scavenger receptors are a broad family of transmembrane multidomain structures classified into six subgroups based on their proposed tertiary structure : SR-A (SR-AI, SR-AII, SR-AIII, MARCO), SR-B (CD36, SR-B1), SR-C, SR-D, SR-E (LOX-1), SR-F (SREC).

The aim of the present study was to investigate the potential expression of SR-A, MARCO, SR-BI, and LOX-1 scavenger receptors at the cutaneous level by means of two models: human keratinocyte cell line HaCaT for which SR expression was studied by immunohistochemistry, flow cytometry, western-blot (WB) and real time PCR analysis; human skin explants for which SR expression was studied by immunohistochemistry and real time PCR analysis.

The present results demonstrate that SR-A, MARCO, SR-BI, and LOX-1 are present in cultured human keratinocyte cell line HaCaT. Furthermore, SR-A, SR-BI, and LOX-1 are also expressed in human epidermis.

026

2,3,7,8-tetrachlorodibenzo-p-dioxin Impairs Differentiation of Normal Human Epidermal Keratinocytes in a Skin Equivalent Model

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Intoxication with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) causes a severe skin pathology in humans called chloracne. Here we tested the effect of dioxin exposure on keratinocytes in an *in vitro* skin equivalent (SE) model in which epidermal keratinocytes (KC) are cultured on a fibroblast/collagen support at the air/liquid interface and differentiate into a stratified epithelium. SEs were exposed to TCDD at concentrations of 10^{-8} and 10^{-7} M in the culture medium for one week. Then they were subjected to histological analysis and Western blotting for differentiation markers. TCDD treatment led to an increase in the thickness of the stratum corneum and to parakeratosis, indicative of an accelerated but incomplete differentiation process. P63 and Id-1, regulatory proteins specifically expressed in non-differentiated keratinocytes, were downregulated by TCDD. While the levels of the spinous layer proteins keratins 1 and 10 were strongly reduced, involucrin was upregulated. Strikingly, TCDD increased the expression of profilaggrin but blocked its processing to filaggrin monomers. Expression of caspase-14, a protease active in terminally differentiated KC, was suppressed by TCDD. Taken together, our data show that TCDD accelerates some early KC differentiation steps but prevents regular completion of the differentiation program in human skin equivalents.

027

Tight Junctions in Psoriasis

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Tight junctions have recently been characterized in normal human epidermis. They are localized to the granular cell layer and contain tight junction proteins such as occludin, ZO-1, and several claudins. Our previous preliminary results have shown that in psoriasis lesion ZO-1 and occludin spread to the acanthotic spinous cell layers (Pummi *et al*, 2001). In order to add understanding of the role of tight junctions in the formation of diffusion barrier in epidermis, we used psoriatic skin as a model.

Nine adult patients with plaque type psoriasis were included in the study which was performed with approval of the joint ethic committee of the University of Turku and The Turku University Hospital. The patients gave their written consent. 3-millimeter punch biopsies were taken from (1) near vicinity of a psoriasis lesion and (2) from a psoriasis plaque. (3) The third biopsy was taken 6-8 weeks later from a healed psoriasis plaque, either after treatment, or from a spontaneously healed plaque. Psoriasis plaque was considered to be healed if there was no scaling but only slight redness or postinflammatory pigmentation. The punch biopsies were frozen in liquid nitrogen and cut into 7 μm frozen sections. The sections were used for indirect immunolabelling for ZO-1, occludin and claudins 1 and 4. All the antibodies were obtained from Zymed.

The results showed that in apparently normal skin next to the psoriasis plaque the distribution of tight junction proteins corresponded to that described for normal skin (Pummi *et al*, 2001; Brandner *et al*, 2002). Specifically, occludin and ZO-1 were restricted to the granular cell layer, while claudins 1 and 4 were distributed to all living cell layers. This indicates that the abnormal distribution of tight junction proteins in psoriasis is induced only in the psoriasis plaque. In the psoriasis plaque, ZO-1 and occludin were detected in a wider zone reaching the middle spinous cell layers, while claudins 1 and 4 were detected in all viable cell layers. However, in some areas, the granular cell layer showed more intense labelling. In healed psoriasis plaque, occludin and ZO-1 were again localized to the upper epidermis. The results indicate that clinical healing of aberrant keratinisation in a psoriasis plaque is associated with morphological normalization of the tight junctions. Furthermore, these results suggest a compensatory role for tight junctions in acanthotic cell layers under conditions when the corneal layer is not fully functioning.

028

A Proteolytic Cascade of Kallikreins in the Stratum Corneum

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We have found three epidermal proteases, stratum corneum chymotryptic enzyme (SCCE, also named human kallikrein 7; KLK7/hK7), stratum corneum tryptic enzyme (SCTE or KLK5/hK5) and kallikrein 14 (KLK14/hK14) present in catalytically active form in the epidermis. All three enzymes, which may be involved in desquamation and skin inflammation, are produced as zymogens, which can be activated by tryptic cleavage. In this study, we produce catalytically active recombinant hK5, hK7 and hK14 in amounts sufficient to allow a further characterisation of the catalytic properties of these proteins. The activity of recombinant hK5 (rhK5), rhK7 and rhK14 was characterised with chromogenic peptide substrates and inhibitors. Both rhK5 and rhK14 were found to have trypsin like primary substrate specificities, but rhK14 was much more efficient towards the substrates tested. rhK5 could auto activate and convert both rpro-hK7 and rpro-hK14 to active forms. The activation of rpro-hK7 was at a low but significant rate with pH optimum around pH 5.6, although the pH optimum for rhK5 activity with chromogenic substrates was alkaline. The rate of activation of rpro-hK14 was faster around pH 6.5. We suggest that the slow activation rate and the acidic pH optimum of rpro-hK7 and rpro-hK14 activation may have physiologic relevance, and propose a model for a cascade reaction of the kallikreins in the epidermis starting from auto-activation of pro-hK5.

029

The Role of β-Catenin in Normal Human Keratinocytes and Involved and Uninvolved Psoriatic Skin

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β-Catenin is a 92Kda protein which has two distinct roles in epidermal cells. As a component of the adherens junction β-catenin has a role in cell-cell adhesion. β-catenin when dephosophorylated is transcriptionally active and acts as a signalling molecule in the wnt signalling pathway. Free β-catenin in the cytoplasm is usually phosphorylated by Glycogen Synthase Kinase 3β (GSK 3β) and targeted for ubiquitination by the proteosome. In response to Wnt signals, GSK-3β is inhibited and activated β-catenin is allowed to translocate to the nucleus where it combines with members of the Tcf/Lef1 transcription factor family to transcribe genes such as cyclin D1 and c-myc which are known to be involved in proliferative responses. Hazan *et al* have shown elevated cytosolic phosphorylated β-catenin in involved psoriatic epidermis and suggested that dysregulation of β-catenin may play a role in regulating keratinocyte proliferation and migration in psoriatic skin. We are interested in the possible effects of un-phosphorylated β-catenin on proliferation and differentiation in psoriasis.

We have investigated the distribution of β-catenin in involved and uninvolved psoriatic epidermis using antibodies against phosphorylated β-catenin and against activated β-catenin. The skin tumour, pilomatricoma caused in most cases by β-catenin mutations, has been used as a positive control. In these experiments we have shown a differential distribution of activated β-catenin in involved and uninvolved psoriatic skin ($n = 6$).

We have used a Tcf/lef luciferase reporter to investigate the transcriptional activity of β-catenin in primary cultures of unselected normal human keratinocytes, cultured in low calcium MCDB153 medium. We have also investigated the role of activated β-catenin in differentiation by examining the effect of β-catenin on a transglutaminase luciferase reporter. Using a β-catenin-GFP construct we have studied the relationship between β-catenin and involucrin.

030

Imatinib Decreases the Basal and Interferon-γ-Induced Expression of the Putative Psoriasis Autoantigen Keratin 17 *in vitro*

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Imatinib, formerly known as ST1571, is a protein-tyrosine kinase inhibitor which is effectively used for the treatment of chronic myelogenous leukemia (CML) and metastatic gastrointestinal stromal tumors (GISTs). In a case of GIST imatinib treatment had been reported to improve concomitantly existing psoriasis. Based upon the observation pointing to a keratin 17 (K17) epitope as possibly relevant psoriasis autoantigen, we have recently proposed a so-called interferon-γ (IFN-γ) / K17 autoimmune loop as a positive feed back mechanism in the etiopathogenesis of psoriasis. We now studied the effect of imatinib in a dose range between $5x10^{-7}$ and $1x10^{-5}$ M using a HaCaT keratinocyte model. A significant reduction of the basal K17 expression and the K17 overexpression induced by 25U/ml IFN-γ was observed for imatinib at concentrations of $\geqslant 2$ and 5 μM, respectively, as compared to the appropriate drug-free controls. When the latter control conditions were set to 100%, the maximum reductions of the basal and IFN-γ-induced K17-expression under 5 μM imatinib were $73.2 \pm 4.4\%$ and $83.5 \pm 15.4\%$, respectively. Thus, our experiments show that imatinib might exert its presumptive antipsoriatic activity at least in part via suppression of psoriasis-relevant K17 overexpression.

031

Sodium Dodecyl Sulfate Increases Sensitivity of Hacat-Keratinocytes to Sidestream Cigarette Smoke *in vitro*

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Sidestream cigarette smoke (SS) can be regarded as a potential toxic influence for skin barrier structures such as membranes and membrane lipids. Therefore the aim of the present study was to investigate *in vitro* whether acute toxic effects of SS on HaCaT-keratinocytes are enhanced by treatment with sodium dodecyl sulfate (SDS) using the neutral red dye release assay (NRRA) that particularly reflects cell membrane alteration and leakage.

For the experiments HaCaT-keratinocytes were incubated 3h with neutral red in a phenol-red free DMEM-medium and then exposed 15 min under PBS to SS that was collected from three research cigarettes (2R4F) immediately before exposure. Finally the cells were destained and the optical density (OD) of the resulting solution was measured at 540 nm. Cells were exposed to 0,2, 0,4, 0,6, 0,8, 1,0 μmol sodium lauryl sulfate (SDS) before smoke exposure, to 0,8 and 1,0 μmol after smoke exposure, and to SS without further treatment. There were also cells which remained untreated.

There was an OD of 0,79 in the untreated control. Exposure with SS decreased OD to 0,71. Treatment with 0,2 μmol SDS alone lead to an OD of 0,77. Increasing concentrations of SDS decreased the OD to 0,59 after treatment with 1,0 μmol. Combined treatment with 0,2 μmol before SS exposure decreased OD to 0,61. Increasing concentrations of SDS decreased the OD to 0,24 after treatment with 1,0 μmol before SS exposure. Treatment with 0,8 μmol SDS after SS exposure decreased OD to 0,43. OD after treatment with 1,0 μmol after SS exposure decreased OD to 0,40.

The results indicate an increasing sensitivity of HaCaT-keratinocytes to SS after treatment with SDS. The sensitivity increases with increasing concentrations of SDS. In consequence it can be concluded that the sensitivity of membrane structures of the skin against SS might be increased not only *in vitro*. Furthermore, even if in a more lesser extent SS also increases sensitivity of the cells to SDS.

032

Clusterin, Vitamin D, and growth regulation of melanoma cell lines

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Clusterin is a glycoprotein that is implicated in various cell functions including cell growth, cell adhesion and apoptosis. There are two known clusterin isoforms that are obtained by alternate splicing, the nuclear (nClu) and the secretory (sClu). The proapoptotic nClu has been shown to be involved in the regulation of cell-cycle progression and apoptosis. We have now analysed expression of clusterin in various melanoma cell lines (e.g. MeWo, SkMel28). All melanoma cell lines analysed revealed strong expression of clusterin mRNA and protein. Interestingly, clusterin mRNA and protein levels were regulated time-dependently by treatment of melanoma cells with 1,25-dihydroxyvitamin D_3. Regulation of clusterin mRNA expression by 1,25-dihydroxyvitamin D_3 was confirmed by measuring clusterin promoter activity (luciferase assay). Our findings indicate that (i) clusterin is strongly expressed in melanoma cell lines, (ii) clusterin expression is regulated time-dependently by 1,25-dihydroxyvitamin D_3, (iii) antiproliferative effects of 1,25-dihydroxyvitamin D_3 on melanoma cell lines may be at least in part mediated via regulation of clusterin expression, (iv) clusterin may be of importance for the growth characteristics of melanoma cells.

033

EDAR Signaling Through EDAR-Associated Death Domain (EDARADD)

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Two recent studies suggest the involvement of a novel death domain adaptor, designated EDARADD/crinkled, in EDAR signaling. EDARADD possesses a C-terminal death domain via which it interacts with the death domain of EDAR. EDARADD has been also reported to interact with different TRAF family member and this interaction is believed to be mediated via its N-terminal domain, which possesses the consensus TRAF-binding sequence Pro-X-Gln-X-Thr. Although the deletion of this motif abolished the interaction of EDARADD with TRAF1,2 and 3 it retained the ability to activate the NF-κB pathway, suggesting that the interaction of EDARADD with the above TRAFs is not needed for NF-κB activation. However, NF-κB activity of EDARADD was abolished upon the complete removal of its N-terminal domain, suggesting that a region other than the above TRAF consensus motif may be responsible for this activity. A recent study has determined the consensus TRAF6-binding motif to be P-X-E-X-X-(Aromatic/Acidic), which we have discovered in the cytoplasmic domain of XEDAR. Although, a consensus TRAF6-binding site is not present in the cytoplasmic domain of EDAR, we have discovered such a site (PVEDTD) in the N-terminal domain of EDARADD. Our hypothesis is that while TRAF6 is recruited directly to XEDAR it may be recruited to EDAR via EDARADD and thus may be involved in NF-κB activation via both EDAR and XEDAR. To test our hypothesis, we begin by testing the recruitment of TRAF6 to EDAR using the technique of co-immunoprecipitation. Second, we generated point mutants of EDARADD in the TRAF2 (aa 34–40) and TRAF6 (aa 17–22) consensus sites and tested their ability to activate the NF-κB pathway and correlated the results with their interaction with different TRAF family members. Finally, we checked the ability of the selected mutants to block EDAR-induced NF-κB in a dominant negative fashion and correlated the results with their effect on the recruitment of TRAF6 to EDAR. Point mutants of EDARADD in the TRAF2 (aa 34–40) and TRAF6 (aa 17–22) consensus sites blocked NF-κB activity. One mutant stabilize the EDARADD protein while the other mutant makes it unstablize. The TRAF binding sites of EDARADD seems to be important as pathogenesis of Hypohidrotic Ectodermal Dysplasia (HED) and ectodermal differentiation. But, EDARADD induced NF-κB activation is complicated and we need additional study.

034

All-trans Retinoic Acid is Dedifferentiating to Human Mast Cells Independent of their Stage of Maturation

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All-trans retinoic acid (ATRA) is a potential master regulator of haematopoiesis, where it primarily affects immature and proliferative leukocytes. Here, we have analyzed the effects of ATRA on human mast cells (MC) that span different stages of maturation, i.e. immature-leukemic HMC-1 5C6 cells, intermediately matured LAD 2 cells, and terminally differentiated skin MC. The expression of typical lineage markers was studied in parallel at the mRNA level by RT-PCR and at the protein level by flow-cytometry. Histamine content was assessed by EIA. ATRA exposure led to a striking downregulation of c-Kit protein expression by all MC subtypes. Moreover, ATRA negatively impacted the expression of the typical MC proteases tryptase and chymase. While neither L-histidine decarboxylase nor intracellular histamine content were affected, protein expression of FcεRI was substantially downmodulated, and this could be attributed to a reduction of FcεRIα and, to a lesser extent, γ specific transcripts. By downregulating most lineage markers, MC are apparently forced to dedifferentiate with ATRA. When protein and transcript levels were compared, however, substantial differences between c-Kit and the proteases were noted. c-Kit downregulation was more pronounced at the protein level, whereas the opposite was found with the proteases. Further analysis revealed the existence of a second mechanism of c-Kit downmodulation that proceeded rapidly and independently of mRNA changes. This pathway was restricted to immature MC and could be mimicked by cycloheximide, suggesting that altered translation may account for the phenomenon. Taken together, our study indicates that MC are significant targets of ATRA throughout their lifespan, but that the molecular events underlying downregulation of lineage markers may be shifted in the course of MC differentiation.

035

Comparison of Human Skin Mast Cells from Two Anatomical Sites – Strongly Diminished Expression of FcεRIα in Breast Skin Versus Foreskin Mast Cells

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Mast cells (MC) are specialized resident tissue cells of hematopoietic origin implicated in allergic inflammation and innate immunity. The heterogeneity of MC is well known, comprising both species-specific and tissue-specific differences. Using MC from two different human skin locations (breast skin and foreskin), we asked in the present study if MC heterogeneity could be further extended to distinct parts of the same organ. When the levels of transcripts that encode MC markers were compared, no difference between foreskin and breast skin MC was detected with the majority of markers. This implies a high degree of stability in MC characteristics irrespective of the precise source of the cells. In strong contrast, however, a substantial and selective decrease of FcεRIα was noted in breast skin versus foreskin MC, while the mRNA for the β chain was only slightly diminished in the former with no detectable difference for the γ chain. This underlines that the genes encoding the three chains of FcεRI are obviously regulated independently. Cell surface expression of the FcεRI protein complex was similar between the two MC types, as evidenced by flow-cytometry, and so was anti-IgE mediated mast cell histamine release, as detected by a histamine analyzer-based detection method or by EIA. This suggests that the level of the α specific transcript in breast skin MC may not be a limiting factor in protein expression and complex formation. Taken together, the study shows that MC heterogeneity exists in the very same organ, but that it is restricted to only certain characteristic elements of the lineage.

036

Withdrawn

037

Infrared Radiation Interferes with the Apoptotic Process by Modulating Early Events of the Mitochondrial Pathway in Human Fibroblasts

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Near-infrared (IR) pre-irradiation protects normal human dermal fibroblasts from ultraviolet (UV) cytotoxicity *in vitro,* and partially inhibits UVB activation of caspase-9 and -3. In the present study we analyse the effect of IR on mitochondria and related apoptotic pathway. As a matter of fact, only few molecules can be chromophores for IR, and cytochromes present within mitochondria are potential candidates.

Human fibroblasts grown in monolayer, or isolated rat mitochondria, were irradiated by IR (1620 kJ/m^2) under conditions permitting to monitor the temperature between 20–25°C, using a 250-W GE 27 IR lamp combined with a Schott RG 715 long-wave pass sharp-cut filter that remove wavelengths shorter than 700nm. Analysis was made using immunochemistry, confocal microscopy and Western blotting.

We firstly observed that IR radiation induced cytochrome c release either in fibroblasts or isolated mitochondria, underlying a key role of mitochondria in the effects of IR.

Further experiments were run with fibroblasts, either on cell crude extracts, or on mitochondrial and cytosolic fractions, to study different elements of the mitochondrial apoptotic pathway. Mitochondrial membrane potential ($\Delta\psi$m) was analysed by flow cytometry.

IR irradiation led to a partial release of cytochrome c and Smac/Diablo (but not apoptosis inducing factor; AIF), a slight but transient decrease in the $\Delta\psi$m, and Bax protein accumulation for up to 24 h. Early apoptotic events in the mitochondrial pathway thus occurred despite a lack of caspase-9 and -3 activation by IR irradiation.

The $\Delta\psi$m then returns to normal, anti-apoptotic proteins (Hsp27, Bcl-2 and Bcl-xL) accumulate, and the level of the pro-apoptotic protein Bax falls.

The arrest of the apoptotic process initially induced by IR radiation could thus be due to a change in the balance between Bax, Bcl-2 and Bcl-xL which was pro-apoptotic during the first 24 h after IR exposure, and became anti-apoptotic. Furthermore the induction of the expression of heat shock protein (Hsp) 27 by IR, which has been shown to inhibit the formation of the apoptosome, can also contribute to this arrest. Together, these actions could aid to prepare the cell to resist to UVB-triggered apoptosis.

In conclusion, this work point out that mitochondria is a key element in the effect of IR and reinforces the fact that at least one IR chromophore is located within mitochondria. This study also indicates that various steps in the mitochondrial apoptotic pathway are modulated by IR exposure. Further work needs to be made on IR-UVB interaction as well as on mutagenesis and DNA repair to evaluate the IR beneficial effects to prevent apoptosis induced by UVB.

038

Localisation of the Melanocortin 1 Receptor (MC1R) within Melanoma Cells Suggests that MC1R is Present at the Melanosome

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The melanocortin 1 receptor (MC1R) is a key regulator of human pigmentation and is expressed on the surface of melanocytes and melanoma cells. Alpha-melanocyte stimulating hormone (αMSH), the ligand for MC1R, is synthesised from pro-opiomelanocortin (POMC) through cleavage of this precursor by pro-hormone convertase (PC)-1 and PC-2. Recent work has shown that αMSH and PC-1 and PC-2 are located within the melanosome (Peters *et al*, J. Invest. Dermatol. 114: 430–437, 2000). The presence of αMSH in the melanosome could imply a role for αMSH independent of MC1R in this organelle, but also raises the issue of whether MC1R is also present at the melanosome. To address this question, we have transfected B16G4F Mc1r-null melanoma cells with human *MC1R* tagged with enhanced green fluorescent protein (EGFP), and have investigated the sub-cellular localisation of MC1R within these cells.

B16G4F cells were stably transfected with wild type (WT) and separately Asp294His variant human *MC1R* tagged at the C terminus with EGFP. αPEP-13 and αPEP-1 antibodies were used to label Pmel-17 protein in early melanosomes and tyrosinase related protein-1 (TRP-1) in late melanosomes respectively. An Alexa-fluor 633-conjugated secondary antibody emitting far red fluorescence was employed for detection in order to ensure there was no crossover into the EGFP excitation / emission spectra by these markers. Images were obtained using laser scanning fluorescence confocal microscopy, and analysed with accompanying Leica software for double positive fluorescence within pixels in Z planes.

Confocal microscopy demonstrated expression of EGFP-WT MC1R and separately EGFP-Asp294His MC1R in a punctate pattern within the cell body as well as at the cell membrane. Co-localisation of WT and Asp294His EGFP-MC1R was observed with both early and late melanosome markers. Co-localisation of EGFP-MC1R was also detected with early and late melanosomes along dendrites following induction of dendricity by culturing in the presence of 10^{-3}M IBMX. These results suggest that MC1R is present close to or within the melanosome as well as at the cell surface, and suggests that part of the function of MC1R may be mediated through its association with the melanosome.

039

Influence of Physiological and Pathophysiological Pericellular Conditions on Endothelial Cell Activation

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Endothelial cells, localized at the interface between blood and tissue play a pivotal role in the process of coagulation and inflammation. Within short periods of time, endothelial cells have the ability to change from a generally anti-coagulatory and anti-inflamatory state to acute endothelial cell activation (ECA). A reliable marker for ECA, is the exocytosis of vonWillebrand-factor (vWF), a strong procoagulatory protein. Potent and well known stimuli in the process of ECA are e.g. histamin or activators of Proteinase-activated receptors (PARs). So far, little is known about the influence of altered physiologic parameters such as hypoxia, temperature and protonconcentration on endothelial cell activation. Therefore in the present study, we tried to assess the role of altered pericellular conditions on ECA. This is important for the understanding of cellular aspects in diseases such as scleroderma, Raynauds-disease and wound-healing, where recurrent ischemic conditions are present. Alterations in skinperfusion lead to reduced levels of oxygen, changes in pericellular temperature and subsequent acidosis. In an especially designed incubating chamber that allows to manipulate pericellular perfusion conditions we were able to determine ECA under physiological and pathophysiological conditions. We found that acute hypoxia leads to an immediate release of procoagulatory vWF. Furthermore, the proinflammatory protein P-Selectin is also expressed on endothelial cell surface. This exocytotic process is mediated by intracellular calcium increase. Hypothermic and acidotic conditions seem to have a protective effect on cell survival by reduced ECA, whereas hyperthermia (fever) significantly increases ECA. We deduce that pericellular conditions are able to inhibit endothelial cells to initiate or enhance inflammatory processess. Therapeutical intervention by altering pericellular conditions is an interesting approach for modifying the local inflammatory, coagulation and perfusion process.

040

Epitope-specific Antibody Response to Mel-CAM Induced by Mimotope Immunization

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Peptide mimotopes of tumor antigen epitopes have been proposed as components of tumor vaccines. In this study we determined the immunogenicity of melcam mim1 and melcam mim2, peptide mimics of an epitope of the melanoma cell-adhesion molecule Mel-CAM. BALB/c mice were vaccinated either with mimotopes or mimotopes coupled to tetanus toxoid. The antibody responses of mice to melcam mim1, melcam mim2 and recombinant Mel-CAM were analysed by an enzyme linked immunosorbent assay and immunoblot analyses. Tetanus toxoid coupled mimotopes led to high titers of IgG mainly of the IgG2a subclass to melcam mim1 and melcam mim2. Immunization with each of the mimotope formulations induced Abs that cross-reacted with recombinant Mel-CAM. Uncoupled mimotopes induced lymphocyte proliferation and cytokine production in spleen cell cultures indicating that both peptide mimotopes also contained T-cell epitopes. Tetanus toxoid coupled mimotopes induced Th1 (IL-2, INF-γ) and Th2 (IL-4, IL-5) cytokines, whereas uncoupled mimotopes induced a Th1 biased T-cell response. Our results suggest that mimotopes potentially represent a novel vaccine approach to induce a tumor antigen specific humoral and cellular response.

041

Integrins and Angiogenesis: Characterization of an Endothelial Cell Growth Suppression Signal

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Angiogenesis is a complex process that takes place physiologically and pathologically during foetal development of some organs, in adult female reproductive cycle, during cutaneous wound healing and tumor development. This process involves steps of proliferation and migration of endothelial cells from pre-existing vessels together with third dimension organisation into mature blood vessels. Our aim is to study the molecular basis of neo-angiogenesis control mediated by the extracellular matrix and its integrin receptors. For this purpose, we analysed an *in vitro* model of angiogenesis, in which primary human endothelial cells harbor either a proliferative or sprouting phenotype depending on the extracellular matrix protein they contact. We observed that laminin, a component of basement membranes, induces a growth arrest signal that overcomes the proliferative effect of fibronectin present in provisional matrices during wound healing. In order to define the signaling pathways involved, and assess the eventual occurrence of specific partners of laminin integrin receptors, we have adapted a yeast two hybrid type of genetic screen applied to transmembrane proteins named "Split-Ubiquitin". This approach has allowed the isolation of several partners, among which the tetraspanin CD9 protein. Our studies demonstrate that this interaction is important for the adhesion dependent control of cell cycle progression in our model of angiogenesis.

042

The Effects of Topical Application of Honey on Wound Healing in Mice: An Experimental Study

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Background: Management of patients with full thickness wound of skin continues to challenge physicians and surgeons in area of cosmetic dermatologic surgery. At the present time, there are some medications that can be used to accelerate the healing of full thickness wound of skin.

Based on studies that have demonstrated improved burn wound healing with honey treatment, we anticipated that honey would accelerate healing of full thickness wound of skin. It is evident from preliminary data, that honey may be an effective, simple, natural, inexpensive and safe therapeutic agent for accelerating wound healing.

Objective. The aim of research proposal is to evaluate the efficacy of honey in accelerating healing of full thickness wound of skin in mice.

Methods. Design: experimental study *Animals:* male N.mRi mice *Sample size:* 6 per study group *Surgery:* two mm full thickness wound of skin in back of mice under general anesthesia *Treatment groups:* Control group: simple dressing, Honey group: daily topical application honey *Study Period:* mice were euthanized on days 4, 7 and 10 post-operation to reflect different phases of wound healing *Assay:* 1- gross pathology of the skin noting presence of infections, dehiscence and repair 2- histological evaluation of the wound site for degree of healing 3-finally the wounds were tested for:

Resilience = ability of the wound to stretch and then resume shape without incurring any tissue damage, ultimate tensile strength = maximum pressure a wound can tolerate before it start to weaken and toughness = total amount of pressure a wound can tolerate before rupturing.

Results. Honey increased: 1) formation of granulation tissue 2) density and activation of fibroblasts 3) keratinization in surface of wound 4) thickness of basement membrane and epidermis 5) thickness of collagen fiber. Honey decreased infection, inflammation, edema and dehiscence.

Finally Honey increased resilience, ultimate tensile strength and toughness of wound in mice.

Conclusion. We conclude that honey accelerates healing of full thickness wound of skin in mice.

043

Expression of Aquaporins in Cells from Human Skin

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Aquaporins (AQPs) are a family of transmembrane channels for water and small solutes. Eleven aquaporins, named aquaporin-0 (AQP0) through aquaporin-10 (AQP10), have been identified in mammals. In human skin, the AQP3 protein is abundant in keratinocytes. AQP1 and AQP5 mRNA were detected in rodent skin and the AQP5 protein was localized to mouse sweat glands. In the present study, we investigated by RT-PCR the mRNA expression of the 11 known human AQPs in a variety of normal human skin cell types including keratinocytes, melanocytes, dermal fibroblasts (HDF), dermal microvascular endothelial cells, white preadipocytes (HWP), monocytes and monocyte-derived dendritic cells (MDDCs). Like in other tissues, we found AQP1 mRNA in dermal endothelial cells. AQP1 was also detected in fibroblasts and melanocytes. In addition to AQP3, AQP10 mRNA was detected in keratinocytes. AQP9 mRNA was found in monocytes and MDDCs, but monocytes also expressed AQP10, whereas MDDCs exhibited AQP3 mRNA. Finally, AQP9 mRNA was also detected in preadipocytes by RT-PCR. AQP expression was not investigated in cells from sweat glands. These results confirm the expression of AQP1 and AQP3 in human skin cells and provides a more comprehensive pattern of expression of these 2 channels in this tissue. In addition, AQP9 and AQP10 mRNA were found in several human skin cell types. Therefore, up to 5 different AQPs (AQP1,3,5,9,10) may be selectively expressed in human skin cells. Whereas AQP1,5 are strictly water channels, AQP3,9,10 belong to the aquaglyceroporin group, also permeable to some small solutes such as glycerol. In the present study we often found mRNA for 2 of the aquaglyceroporins per cell type. The physiological consequences of this distribution of aquaporins will now be investigated.

044

IL-3 Induces Expression of Lymphatic Phenotypic Markers

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Factors determining lymphatic differentiation in the adult organism are yet not well characterized. We have made the observation that mixed primary cultures of blood (BEC) and lymphatic (LEC) endothelial cells grown under standard conditions change to a 100% lymphatic phenotype during subculture. After passage 6 they uniformily express LEC-specific markers Prox-1 and podoplanin. We show that LEC but not BEC constitutively express IL-3 and that IL-3 is responsible for the differentiation of BEC into LEC. Using sorted BEC, IL-3 induces Prox-1 and podoplanin expression and expression persists after subsequent withdrawal of IL-3. Using sorted LEC, blocking IL-3 activity by IL-3R alpha chain antibodies results in a loss of Prox-1 and podoplanin expression. To analyze the situation *in vivo*, IL-3 was injected into melanomas subcutaneously grown on the backs of SCID mice. In this model, IL-3 significantly increased numbers of podoplanin positive vessels, which were all positioned within the lymphatic vascular tree, whereas numbers of blood vessels remained constant. In conclusion, IL-3 is a novel factor capable of inducing lymphatic differentiation.

045 [Oral 038]

Fibroblast Growth Factor-2 Effects on Wnt Target Genes in Endothelial Cells

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Beta-catenin is a component of the Wnt signaling pathway, it transduces signals to Lef/Tcf transcription factors and upregulates genes like cyclin D1. Recently we found that in human endothelial cells beta-catenin is translocated into the nucleus upon stimulation with FGF-2. Increased nuclear beta-catenin leads to upregulation of cyclin D1 promoter expression in a Lef/Tcf dependent fashion. EMSA analysis demonstrates binding of Tcf-4 and beta-catenin to the Lef/Tcf binding site in the cyclin D1 promoter. We next analyzed effects of FGF-2 on matrilysin, which is thought to be another target of the Wnt pathway. As seen with cyclin D1, FGF-2 induces matrilysin promoter activity and mRNA expression. In contrast to cyclin D1, this FGF-2 effect is Lef/Tcf independent: There is only negligible Tcf-4 binding to the two Lef/Tcf sites in the matrilysin promoter, beta-catenin overexpression or mutations of the Lef/Tcf sites do not alter FGF-2-induced promoter activity. In contrast, overexpression of a dominant negative Stat3 mutant significantly reduces FGF-2-induced matrilysin promoter activity. This is no direct Stat3 effect, because we could not detect Stat3-binding to several putative Stat3-binding elements. The dominant negative Stat3 mutant appears to inhibit matrilysin promoter activity indirectly by inhibiting AP-1-related gene transcription. In conclusion, although both, cyclin D1 and matrilysin are thought to be Wnt/Lef/Tcf targets, in endothelial cells FGF-2 induces only cyclin D1 but not matrilysin in a Lef/Tcf dependent manner.

046 [Oral 058]

TNF/TNFRI-mediated Psoriasiform Dermatitis in Conditional IkappaB-alpha-deficient Mice Depends on Ubiquitous Inactivation of IκB-α

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The inhibitor of NF-κB, IκB-α, is degraded following phosphorylation by the IκB kinase (IKK) signalosom, thus inducing activation of NF-κB. Several studies suggested a role for NF-κB and IκB-α in epidermal differentiation. Nevertheless, the exact function of NF-κB and IκB-α in the epidermal skin compartment remains under discussion. Here we used Cre/loxP-mediated gene targeting in order to investigate the function of IκB-α in the epidermal compartment. Newborn mice with a ubiquitous deletion of IκB-α (*ikba*$^{\Delta/\Delta}$) develop an inflammatory skin disease with a psoriasis-like phenotype. The skin disease is tumor necrosis factor/TNFRI-mediated. In contrast, specific deletion of IκB-α in epidermal keratinocytes utilizing K5-Cre resulted in hyperproliferation without inflammation. Moreover, deletion of IκB-α in T-cells, monocytes or neutrophilic granulocytes neither perturbed epidermal homeostasis nor induced inflammation. Our results suggest, that IκB-α is necessary for the regulation of epidermal keratinocyte response to exogenous inflammatory stimuli and epiderimal homeostasis.

047

Stroma-Mediated Dysregulation of Liver and Bone Marrow Myelopoiesis in Mice Lacking IκB-α

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During perinatal development, hematopoietic maturation occurs in the liver and the bone marrow. Newborn mice with a ubiquitous deletion of IκB-α (*ikba*$^{\Delta/\Delta}$) develop a severe hematological disorder characterized by a hypergranulopoiesis with an increase of CFU-GEMM. Here we report that the development of this particular myeloproliferative disturbance is mediated by continuous perinatal expression of Jagged1 in IκB-α-deficient hepatocytes resulting in a permanent activation of Notch1 in neutrophils. In contrast, in mice with a specific deletion of IκB-α only in the myeloid lineage (*ikba*$^{my\Delta/my\Delta}$) and in fetal liver cell chimeras (*ikba*$^{FL\Delta/FL\Delta}$) a cell-autonomous induction of the myeloproliferative disease was not observed. Co-culture of IκB-α-deficient hepatocytes with wild-type bone marrow cells induced a Jagged1-dependent increase in CFU-GEMM. In summary we show that a pre-malignant hematopoietic disorder with consecutive transformation can be initiated by non-hematopoietic cells.

048

Regulation of Epidermal Tight Junctions During Staphylococcal Skin Infection

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Tight junctions (TJ) play a central role in epithelial barrier function. Recently, their proteins such as claudins, occludin and ZO-1 were identified within the epidermis. Although TJ proteins are known as targets for bacteria and their toxins in simple epithelia and the skin being a major target of microbial assaults, there is no data available about the regulation of TJ proteins during skin infection. Therefore, we investigated the influence of the pathogen *Staphylococcus aureus* and the commensal *Staphylococcus epidermidis* on epidermal TJ expression. We established a porcine skin infection model: punch biopsies were infected for 24 hours with *Staphyloccus aureus* (w54 and wt) and *Staphylococcus epidermidis* (1457) being in the postexponential phase. Subsequently, expression of the TJ proteins claudin 1, ZO-1 and occludin were determined by immunohistochemical stainings. We found, that compared to the control group, *S. aureus* induced a down regulation of claudin 1 and ZO-1, whereas *S. epidermidis* exerted no such effects. With respect to the expression of occludin we observed no alterations after staphylococcus infection. TUNEL stainings revealed a slight, non significant up-regulation of apoptosis within infected compared to control skin. These results demonstrate for the first time that *S. aureus* induces down-regulation of TJ proteins and suggest that these effects may be relevant during skin infection.

049 [Oral 037]

Hair Follicle Nestin-Expressing Stem Cells Give Rise to Nascent Blood Vessels in the Skin

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We have previously demonstrated that the neural-stem cell marker nestin is also expressed in hair follicle stem cells in transgenic mice as visualized by nestin-driven green fluorescent protein (GFP). Since it is known that blood vessels can express nestin, the present study was designed to determine if blood vessels in the skin were related to hair follicles. In the nestin-GFP transgenic mice, the hair follicles are linked by a network of nestin-GFP positive vessels. When a red fluorescent protein (RFP)-expressing B16 melanoma was transplanted to the nestin-GFP mice, nestin-expressing vessels grew from the nestin-expressing stem cells hair follicle bulge area into the growing melanoma. Fibrissa hair follicles were transplanted from nestin-GFP C57BL6 mice to nude mice. After transplantation, nestin-GFP was visualized by fluorescence in nascent vessels growing from the transplanted hair follicles in the nude mouse skin. Nestin-GFP hair follicles were also observed to give rise to GFP-expressing vessels in RFP-expressing B16 melanoma after both were transplanted to nude mice. Nestin-expressing vessels were also visualized by GFP fluorescence growing from hair follicles in healing wounds in the nestin-GFP mice. Immunohistochemical staining showed that the endothelial-cell-specific markers CD31 as well as von Willebrand factor (vWF) and nestin colocalize in the nestin-GFP-expressing nascent vessels both in the nestin-GFP mice and in nude mice transplanted with nestin-GFP-expressing vibrissa hair follicles. The data thus suggest har follicles give rise to blood vessels in the skin. This model enables very early events in skin angiogenesis, including skin-tumor angiogenesis to be visualized, and to used for antiangiogenesis drug screening. The results of the present study suggests the pluripotency of hair follicle nestin-expressing stem cells which can form blood vessels as well as much of the hair follicle structure and epidermis.

050

Control of the Intracellular Localization of CD1e

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CD1b and d are cell surface expressed, and cycle between the plasma membrane and endosomes. Their localization in endosomes is controlled by tyrosine motifs in their short cytoplasmic domain, which bind AP3. In contrast, CD1e accumulates in Golgi compartments, then reaches CD1b + late endosomes, where it remains in a soluble form, due to a proteolytic cleavage. Its long cytoplasmic domain does not display known targeting motifs. We explored how the transport of CD1e is controlled. Deletion and substitution mutants were engineered and expressed in normal and AP-deficient cells. We demonstrated that the cytoplasmic domain is sufficient to actively target CD1e towards late endosomes, using an AP3-independent pathway. In contrast, a role of AP-1 in the biosynthesis of CD1e could be demonstrated. Deletion mutants revealed a role of different parts of the cytoplasmic domain in the retention of CD1e in TGN, and its intracellular retention, facilitating its routing towards late endosomes. Mutations that allowed cell surface expression of CD1e, including the substitution of the cytoplasmic domain by artificial sequences, did not abrogate the targeting towards endosomes. A 27kD protein co-immunoprecipitated with CD1e when the acidification of endosomes was blocked by bafilomycin. Experiments showed that the cleavage of CD1e in endosomes only occurs when it associates with p27. Thus, CD1e reaches late endosomes and is not expressed on the plasma membrane, using a cellular pathway not shared by other CD1 molecules. This distinct pathway may provide an additional means of immunological surveillance of cellular compartments by an AP3 independent mechanism.

051

The Murine Dermis CONTAINS Cells with *in vitro* Clonogenic Potential

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Evidence exists that the mammalian dermis contains multi- or pluripotent stem cells (SC) with the capacity to differentiate into various cell types. The phenotype of these SC is still unclear. We wanted to determine if cell-surface markers of hematopoietic stem cells (HSC) as Sca-1, CD117 and CD34 can be used to identify and enrich dermal SC. FACS analysis of freshly isolated newborn and adult dermal mouse cells revealed that a small portion of $CD45^+$ cells co-expressed Sca-1 and CD117 molecules, suggesting the presence of a small reservoir of stem cells. When testing the *in vitro* clonogenic capacity of unpurified newborn and adult dermal cells in methylcellulose supplemented with cytokines inducing hematopoietic differentiation, we identified different colonies, including adipocytes and sebocytes as determined by red oil and K14 staining, respectively, and colonies with a myeloid phenotype (e.g. toluidine blue positive mast cells). In contrast, culturing peripheral blood cells under identical conditions failed to generate colonies. The culture of lineage-depleted newborn dermal cells revealed that most outgrowing colonies were of adipocyte and sebocyte phenotype. Using a plate-bound technique, lineage-depleted dermal cells were further enriched for markers present on HSC. The vast majority of purified $Sca\text{-}1^+$ (98%) cells were $CD34^+$ and failed to express CD45 molecules. These cells gave rise only to adipocytes and sebocytes. This work is the first to demonstrate a strategy to phenotype and highly enrich cells from the dermis with clonogenic capacity.

052

K1b - a New Member of The Human Type II Keratin Gene Family is Specifically Expressed in Eccrine Sweat Gland Ducts

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The recent completion of a reference sequence of the human genome now allows a complete characterization of the type II keratin gene domain on chromosome 12q13.13. This, domain, ca. 790 kb in size, contains 26 keratin genes and 8 pseudogenes. 23 of these genes and four pseudogenes have been previously described and therefore this domain comprises 3 new functional genes: *K1b*, *K5b*, and *K6l*. Northern analysis of the keratins in mRNA of major organs as well as in specific epithelial subtypes shows singular expression of these keratins in skin, hair follicle and tongue, respectively. The expression of K1b is characterized in this study. Using specific 3'-probes for *in situ* hybridization and specific antibodies K1b for indirect immunofluorescence, K1b is specifically expressed in the eccrine sweat gland ducts. In more detail, as these ducts are stratified in two layers this protein is detectable in the luminal layer only. The glandular part of this appendage is negative. Moreover, other glands and their ducts investigated including e.g. apocrine sweat glands, mammary glands, salivary glands, Bartolini glands or sebaceous glands were also negative.

In tumors of eccrine sweat glands K1b is a suitable "marker" for the characterization of the origin and state of differentiation of these neoplasms.

053

Membrane Estrogen Receptor Mediates Acute Nitric Oxide Release in Dermal Endothelial Cells

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It is known that estrogens play an important role in regulating vascular endothelial function. This effect is mediated by the two estrogen receptors: ERα and ER β via classic genomic pathway involving nuclear estrogen receptors (ERα and ER β) and also nongenomic pathway involving a plasma membrane estrogen receptor (ERα). This estrogen vascular effect has been well determined for endothelial cells derived from large and small vessels but the estrogen effect on human dermal microvascular endothelial cells (HDMECs) function is not documented. In the present study, by Western blot analysis, we showed that HDMECs expressed both estrogen receptors (ERs): 66 kDa- ERα and 59 kDa- ER β proteins. By fluorescence microscopy using ERα specific antibody, we found immunoreactive sites on plasma membrane of nonpermeabilized HDMECs in a distribution similar to that reported for human umbilical vein endothelial cells (HUVEC) while permeabilized cells exhibited intense nuclear fluorescence. This demonstrated the presence of ERα as a membrane associated as well as nuclear receptor in HDMECs. In contrast, using ER β specific antibody, fluorescence microscopy revealed only nuclear staining. Furthermore, treatment of HDMECs with estradiol -17 β (E2) at physiologic concentration (10^{-9} M) and with the membrane impermeable E2-BSA rapidly induced nitric oxide (NO) release with maximum induction (2.5 to 3 fold) within 10 min of treatment. These results suggested that release of NO in HDMECs by estrogens is mediated via estrogen receptor localized on the plasma membrane. Since NO is known as a critical mediator of angiogenesis, this study supports the angiogenic activity of estrogens on HDMECs.

054

Differential Interaction of Tight Junction-Proteins with Inflammatory Cells in the Epidermis

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Tight junctions (TJ) are occluding cell-cell junctions which are known to form and maintain barriers in simple epithelia and endothelia. They have also been identified in murine and human skin and various reports suggest that they play an important role in barrier function of the epidermis too. TJ proteins are known to interact with inflammatory cells in simple epithelia and endothelia, but nothing is known regarding the epidermis.

We investigated TJ proteins, i.e. claudins 1 and 4, occludin, JAM-1 and protein ZO-1, in inflammatory skin diseases by using immunofluorescence microscopy. Moreover we investigated adherens junction- and desmosomal proteins in comparison. In psoriasis we observe a downregulation of TJ proteins as well as of desmosomal and AJ proteins in areas with transmigrating inflammatory cells. The cell-cell junctions seem to reseal after inflammatory cells passed through. In lichen ruber and eczema a downregulation of cell-cell junction-proteins was only found in cells with direct contact to inflammatory cells. Referring to these results we discuss the putative differential influence of neutrophils and lymphocytes on cell-cell junctions during inflammatory diseases and their role in pathogenesis.

055

A Scanning Electron Microscopic Study of the Effect of Some Hair Care Products on Human Scalp Hair Shaft

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The general purpose of hair care products and hair styling manoeuvers is to restore the natural beauty of hair, giving it volume, softness and shine. However, a large number of these proucts fail to achieve their purposes. To the contrary, they may exaggerate the damage induced by environmental factors. The present work aims at throwing a close view on the surface appearance of human scalp hair shafts exposed to some hair care products. It also aims at investigating the effect of these factors on the normal trace element content of hair. Samples from human scalp hair shafts were exposed to certain commercial hair shampoo, gel, conditioner, hair bleacher as well as subjected to perm and fair processes. The treated samples were examined by the scanning electron microscope and subjected to X-ray microanalysis for quantitative estimation of the elementary concentrations compared to control samples. The normal overlapping appearance of the cuticle and the clefts between the cuticular scales were disturbed particularly in hair shafts exposed to gel, fair, perm and hair bleacher. Hair straightening using the fair revealed the gravest insults to the hair not only through the structural damage but also by causing burning of the hair cuticle and deformity of the hair cylinder. X-ray computer analysis of the hair elements revealed that sulphur, cupper and zinc were most affected. Correlation of the quantitative elementary results with the morphological changes of hair shafts could explain the mechanism of action of such hair care products in damaging the human hair.

056

TNF-α and IL-1β Stimulation of HepG2 Cells Leads to a Time Dependent Shift in the Composition of the NF-κB Dimers and in the NF-κB Binding Affinity

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The purpose of this study was to determine the composition of the NF-κB dimer and the NF-κB binding activity to IL-8 and p53 promoters in TNF-α or IL-1β stimulated Hepato-carcinoma cells (HepG2 cells) as a function of time. Lawrence *et al* (2001) showed that NF-κB activation is essential for both initiation and resolving inflammation. Kunsch *et al* (1992) showed that the specific sequence of the κB binding motif in the promoter region of a gene determine which NF-κB dimer/dimers that can bind and initiate or inhibit transcription.

HepG2 cells were stimulated with either IL-1β (20 ng/ml) or TNF-α (50 ng/ml) and then harvested at different time points (0–72 h). DNA-protein binding of nuclear extract was analyzed using EMSA with DNA oligo sequences identical to the NF-κB binding of the human IL-8 and p53 promoters. Supershifts were performed with 〈p50 and 〈p65 antibodies. Transcriptional activity of p53 and IL-8 genes were analyzed by a reporter gene assay (using plasmid constructs containing the NF-κB binding elements from either the IL-8 or p53 promoters). Protein expression was determined by western blotting using αp50 and αp65.

The reporter gene assay showed an activation of the IL-8 reporter gene which was maximal (20 fold) after 3 hours of stimulation with TNF-α or IL-1β. Then it declined and reached basal levels after 12 hours. The p53 reporter gene expression was also maximal (5 fold) after 3–6 h stimulation. Interestingly the decline in p53 gene expression was much more slow than IL-8. It hadn't reached basal levels after 72 hours of stimulation. The difference in activation between the IL-8 and p53 plasmid after TNF-α or IL-1β was highly significant ($p < 0.05$).

EMSA's revealed a difference in the composition of the NF-κB dimers binding to the IL-8 κB and p53 κB binding motif. The IL-8 κB binding motif binds the p65:p65 homodimer and p50:p65 heterodimer, whereas the p53 κB binding motif, binds the p50:p50 homodimer and the p50:p65 heterodimer. The p50:p65 heterodimer showed no significant difference in the binding activity to either the IL-8 or the p53 oligo. The induction was biphasic with a peak after 15–30 min and a new peak 3-6 hours after stimulation. The increased binding activity of the p65:p65 homodimer seemed not to last more than 1 hour, whereas the increased binding activity of the p50:p50 homodimer seemed to be sustained throughout the 72 hours study period. Western blot of nuclear extracts confirmed that the p65 subunit was removed faster from nucleus than the p50 subunit over the 72 h study period.

Because the IL-8 and p53 reporter plasmids only contained the NF-κB binding sequence of the promoters the prolonged activation of the p53 plasmid compared to the IL-8 plasmid is regulated by NF-κB. Our data also demonstrate that the time dependent difference in NF-κB binding to the IL-8 and p53 κB binding motif is due to a change in the composition of the NF-κB dimers. In the initial phase of NF-κB activation after IL-1β or TNF-α stimulation p50/p65 and p65/p65 are the dominating dimers whereas the p50/p50 homodimers dominates in the latter phases.

057

Identification of Aminopeptidase N (APN/CD13) as a New Target to Regulate Sebocyte Growth, Differentiation and Cytokine Production *in vitro*

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The ectopeptidase aminopeptidase N (APN, CD13, E.C.3.4.11.2) is expressed on endothelial cells, cells of the myelo-monocytic lineage, activated T cells, fibroblasts and keratinocytes. In addition to its proteolytic function it is involved in the regulation of various physiologic functions such as proliferation, cytokine production, cell-cell interaction and angiogenesis. In this study we demonstrate for the first time that APN/CD13 is present on human SZ95 sebocytes and is involved in the regulation of proliferation, differentiation and IL-1RA production *in vitro*.

By RT-PCR, flow cytometry, immunohistochemistry and Ala-pNA hydrolysis we found a high expression of APN on the mRNA, protein and enzyme activity level (CD13 expression 100%, APN activity 205 $\pm$ 54 pkat/10^6 cells). The APN inhibitors Actinonin and Bestatin suppressed the enzymatic activity of SZ95 sebocytes in a dose-dependent manner. After 24 h of incubation with these inhibitors, the DNA synthesis, measured by ^{3}H-thymidine incorporation, was significantly and dose-dependently reduced (Actinonin IC25 2.5 μM, IC50 15 μM; Bestatin IC25 3 μM). The differentiation of SZ95 sebocytes measured by nile red fluorescence intensity was increased after 48 h of incubation with different inhibitor concentrations. Furthermore, the inhibitors significantly enhanced the production of IL-1RA mRNA and protein as detected by ELISA in SZ95 sebocyte supernatants and RT-PCR by 1.4–2.6 fold.

Our data provide evidence that Aminopeptidase N represents a new target for the regulation of sebocyte proliferation, differentiation and cytokine production. Therapeutic application of APN inhibitors maybe beneficial in acne and/or other diseases with sebaceous hyperproliferation.

058

Localized MMP2 Secretion Induced by Melanoma Epithelial Cell Interaction

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Matrix metalloproteinases (MMPs) are known to play a crucial role in tumour cell invasion. In previous experiments using the immunofluorescence technique we could show that human melanoma cells store MMP2 in small vesicles associated with the microtubule system. Co-localisation studies using MMP2 and kinesin specific antibodies showed that 81.9 $\pm$ 2.3% ($n = 5$) of all MMP2 containing vesicles are associated with kinesin. The motor protein kinesin is known to transport cargo along microtubules towards its plus end, indicating that these vesicles are conveyed along microtubules by kinesin. Destruction of microtubule function by paclitaxel leads to an impaired secretion of MMP2 and MMP9 and diminishes invasive properties of melanoma cells. Moreover paclitaxel treated MMP2 vesicles were found to accumulate in the perinuclear region as observed by immunofluorescence microscopy. In a further step MMP2 exocytosis during cell-cell interaction with epithelial MDCK-C7 cells was investigated. For this purpose we co-cultivated both cell lines and stained surface-localized MMP2 on the plasma membrane of the non permeabilized cells. We found MMP2 concentrated in those areas of melanoma cells that were in close neighbourhood of epithelial cells. Via atomic force microscopy we visualized cell surface topography. Perfectly in line with the immunofluorescence studies this technique revealed pore-like structures indicating fusion events of exocytotic vesicles. These pores were predominantly found in those areas of melanoma cells that were in direct contact to epithelial cells.

We conclude: i. Interaction between melanoma cells and epithelial cells stimulates localized MMP2 release. ii. Paclitaxel reduces exocytosis of MMP2 and MMP9 and thus diminishes invasive properties of human melanoma cells.

059

The Hair Follicle as a Source and Target of Melatonin

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The aim of this study was to investigate a) whether hair follicles are peripheral melatonin targets (i.e. express membrane melatonin receptors [MT1, MT2] and a mediator of nuclear melatonin signaling, RORα), b) whether melatonin exerts functional effects on murine and/or human hair follicles *in situ*, and c) whether the hair follicle is an extrapineal site of melatonin synthesis. Real time PCR for MT1, MT2 and RORα revealed that MT2 and RORα are transcribed in murine skin in a hair cycle-dependent manner. By immunohistology, RORα like immunoreactivity was detected in dermal papilla and hair follicle keratinocytes in a hair cycle-dependent manner. Functionally, melatonin (0.01 to 1 nM) significantly inhibited the follicular keratinocyte apoptosis in short term-mouse skin organ culture. Furthermore, melatonin decreased gene and/or protein expression of estrogen receptors in mouse and human hair follicle *in vitro*. By immunohistochemistry, melatonin-like immunoreactivity was found in the outer root sheath of murine and human hair follicles. Liquid chromatography/mass spectrometry and radioimmunoassay revealed that norepinephrine (i.e. the key stimulus for melatonin synthesis in the pineal gland) substantially increased the melatonin content of murine skin and human hair follicles *in vitro*. In conclusion, we provide evidence that murine and human hair follicles are prominent targets for melatonin bioregulation through MT2 and RORα, whose stimulation also affects the well-appreciated hair growth-regulatory receptors (here: downregulation of estrogen receptors), and that the hair follicle is also an extrapineal source of melatonin. These evidences indicate that melatonin may play a role in the endogenous controls of hair follicle cycling.

060

Characterization of Kallikrein Activities in Human Stratum Corneum

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The overall aim of this study was to further characterize the activity from three human kallikreins in stratum corneum, among them human kallikrein 14 (hK14), a kallikrein previously not known to exist in epidermis. In our group, two epidermal serine proteases, hK5 (SCTE) and hK7 (SCCE), have been cloned and charachterized. They are believed to be involved in stratum corneum turnover through a proteolytic cascade ending in the breakdown of desmosomal proteins. It was found that a major part of the trypsin-like activity, measured by cleavage of chromogenic peptide substrates, in extracts from plantar stratum corneum, could not be ascribed to hK5. In search for yet another serine protease we have now purified hK14 from human plantar stratum corneum through reversed phase chromatography. A panel of nine individuals were subjected to tape stripping of superficial non palmo-plantar stratum corneum. Protein contents of tape strips were extracted. Western blot of extracts revealed the presence of hK14 in all individuals tested. Quantification experiments of the relative amounts of hK5, hK7 and hK14 in human epidermis showed that the enzymes are present in a ratio of approximately 3:10:1. α1-antitrypsin inhibitor analysis showed that the major part of hK14 in human stratum corneum is present in active form. We have also noted that active hK5, hK7 and hK14 are present in sweat. This finding might have relevance for patients suffering from atopic dermatitis as these often experience a more pronounced itch when sweating. We hereby conclude that hK14 is present in human stratum corneum. hK14 is a major contributor to the trypsin-like activity in human stratum corneum, indicating that hK14, in addition to hK5 and hK7, is likely to have an important function in the physiology of human stratum corneum.

061

Glycosaminoglycans and Skin Repair

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The aim of this study was to precise the role of GAG in wound healing which consists in precise processes encompassing a set of complex interlocking and overlapping phases, including inflammation, angiogenesis, matrix deposition and epithelialization. These processes are mediated by interacting molecular signals such as cytokines and growth factors. Matrix-generating fibroblasts contribute to lay down and remodel their new own matrix components. Glycosaminoglycans moieties (GAGs) of proteoglycans play important roles in tissue healing by their capacity to interact with enzymatic proteins and to store heparin binding growth factors (HBGF). We have used engineered polymers called Re-GeneraTing Agents (RGTAs) as they stimulate *in vivo* tissue repair. These compounds mimic the protecting and potentiating effects of heparan sulfate (HS) toward HBGF and modulate enzymatic activities of inflammatory and matrix remodelling phases.

This experimental study was performed using a standardised doxorubicin-induced skin necrosis in mice. This model reproduces extravasation effects of chemotherapeutic agents, which occur in the most frequent complications in cancer patients. The GAG-mimic compound used was RG-1250b, a carboxymethyl O-sulfonate dextran (Mr 85 000; degrees of substitution of carboxylate (0,53) and *O*-sulfonate (1,24) per glucosidic unit. RG-1250b in saline buffer was administered, day eleventh after doxorubicin-intradermal injection corresponding to the maximum of necrotic area development, simultaneously weekly by intra-muscular way (1,5 mg/kg) and, topically each two days (0.1 mg/mL).

Two days after RGTA treatment, a very significant increased of ulcer enclosure (50% of original area) is observed as compared to control group (less than 10%). Collagen I, III and V biosynthesis were measured by *ex vivo* (^{3}H)proline incorporation in necrotic skin biopsies. In untreated ulcerated skin, relative collagen III proportion was largely amplified, reflecting fibrosis-related alterations. RG-1250b restored collagen III biosynthesis to control values. The quantitative evolution of HS and chondroitin sulfate (CS) were determined during formation and enclosure of the skin ulcer by the DiMethylMethylene Blue method, with and without nitrous acid treatment. In healthy skins, CS and HS represented respectively 20% and 80% of total GAG quantities. A dramatic decrease of GAG content was observed during skin ulceration followed by a rapid two-fold increase detected at the starting point of spontaneous skin repair. Normal GAG levels were restored eighteen days after ulcer induction. RG-1250b treatment showed to enhance skin healing by restoring GAG content after only two days of treatment. RG-1250b modulates GAG synthesis and especially HS synthesis. Transcript's expression of several enzymes involved in HS metabolism, including EXT-1 and 2, NDST-1 and 2, 2-OST, 6-OST, 5-epimerase and heparanase by real-time RT-PCR, are currently studied.

In conclusion, RG-1250b exhibits powerful anti-fibrotic and regenerative activities on ulcered skins. RGTA constitute a new class of puissant therapeutics agents in the treatment of skin ulcers and can be used as tools to better understand physiological role of endogenous GAG.

062 [Oral 012]

Beta-endorphin Modulates Lipogenesis in Human Sebocytes

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Previous research in our laboratories has demonstrated that the human sebaceous gland is a target organ for several stress hormones including alpha-melanocyte-stimulating hormone (alpha-MSH) and corticotropin-releasing hormone (CRH). To further investigate the role of stress hormones in acne, we examined the expression of opioid receptors (OR) and the biological actions of beta-endorphin (beta-ED), a natural high-affinity ligand for the mu-OR (MOR) and low-affinity ligand for the delta-OR (DOR), in human sebocytes. RT-PCR, Western immuno-blotting and immunofluorescence studies identified the MOR but not the DOR in the human sebocyte cell line SZ95. Expression of the MOR was also confirmed in the sebaceous gland in normal human adult skin and was detectable primarily in peripherally located sebocytes. SZ95 sebocytes did not express the beta-ED precursor pro-opiomelanocortin (POMC) as shown by RT-PCR analysis and Western immunoblotting and stimulation of cells with prototypical POMC inducers such as tumor necrosis factor-alpha, alpha-MSH, dbcAMP, phorbol ester and CRH failed to induce POMC. On the functional level beta-ED significantly suppressed the growth of SZ95 sebocytes induced by epidermal growth factor in chemically defined calcium-rich medium. On the other hand, beta-ED enhanced lipogenesis as shown by Nile red staining and gas chromatography. Accordingly, beta-ED dramatically increased the amount of C16:0, C16:1, C18:0, C18:1 and C18:2 fatty acids in an extent similar to linoleic acid used a positive stimulus. Our data show that human sebocytes express the MOR and respond to beta-ED with reduced *in vitro* proliferation and increased lipogenesis. Our data further demonstrate another link between stress and acne at the biochemical level.

063

Carnitine is Synthesized in the Epidermis and Supplies the Energy Necessary for Epidermal Permeability Barrier

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Carnitine is an essential cofactor in the transport of long-chain fatty acids across the inner membranes of mitochondria. As such, carnitine, plays an important role in the supply of energy via β oxidation. Carnitine homeostasis in mammals is maintained by both endogenous synthesis and exogenous intake from dietary sources. The endogenous synthesis takes place exclusively in the liver, kidney, and brain, while other depend on active uptake via carnitine transporter. Relatively little is known about the role and biosynthesis of carnitine in the skin. The aims of this study were as twofold: 1) to clarify the importance of carnitine by elucidating its synthesis in epidermis; 2) to clarify the role of carnitine in the supply of energy via β oxidation, a process requisite for the epidermal permeability barrier, one of the most important epidermal functions.

Two findings indicated that carnitine may be synthesized in keratinocytes. Firstly, reverse transcription polymerase chain reaction (RT-PCR) confirmed the expression of three enzymes for carnitine biosynthesis in cultured normal human keratinocytes (trimethyllysine hydroxylase, trimethylaminobutyraldehyde dehydrogenase, butyrobetaine dioxygenase). Secondly, ^{14}C-carnitine synthesis was observed in keratinocytes cultured with ^{14}C-lysine. Carnitine culture also significantly increased the β oxidation in keratinocytes, and treatment with carnitine increased the synthesis of intercellular lipids in a skin-equivalent model.

In the last stage of our study we investigated the efficacy of topical application of carnitine with dry skin volunteers. Transepidermal water loss (TEWL) was significantly decreased when a cream containing carnitine was topically applied to the shins of the dry skin volunteers for 6 weeks. In tests performed after stripping the lipids from the stratum corneum by acetone-ether treatment, TEWL was lower in skin treated with the carnitine cream than in skin treated with vehicle (placebo cream). It thus appeared that the topical application of carnitine suppressed the increase of TEWL induced by the acetone-ether treatment. The formation of epidermal permeability barrier was apparently accelerated following the increase of β oxidation of the epidermis with the topical application of carnitine.

These observations confirmed that carnitine plays an important role in epidermis and supplies the energy necessary for epidermal permeability barrier via β oxidation.

064 [Oral 054]

Nuclear Microscopy and Electron Microscopy Studies of Percutaneous Penetration of Nanoparticles in Mammalian Skin

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The ion microbeam techniques, Scanning Ion Transmission Microscopy (STIM), developed in our laboratory were associated with Particle Induced X-ray Emission (PIXE) in order to determined the presence and content of mineral ions and exogenous elements in the different strata of the skin of several mammals: mice, pig and human. PIXE and STIM were associated with Transmission Electron Microscopy to implement this analysis. PIXE and STIM allow respectively to map most elements present in epidermal sections, which appeared highly compartmentalized in the different skin strata, and to image the skin structure on the basis of its density contrast. Micro-PIXE analysis allows the simultaneous mapping of a dozen of elements (Na, Mg, S, Cl, K, Ca, Ti, Zn…) in tissues sections. STIM delivers images of tissues with sub-micrometer resolution, both rapidly and non destructively, authorizing localization of cellular structure. In addition, it allows the measurement of the sample mass to normalize X-ray data in terms of concentration. STIM method may be applied on un-embedded sections, cryofixed and freeze-dried. This sample preparation is undoubtedly an advantage when addressing percutaneous penetration studies. In new solar cream, nanoparticles of TiO_2, ZnO, Al_2O_3, are included to provide protection againts UV. The aim of this work is to determine the potentiality of percutaneous penetration of such particles. We focused our study on formulations containing solely TiO_2 nanoparticles. When such formulations were applied on skin, TiO_2 particles were solely detected in the intercellular spaces between the corneocytes of the outermost layers of the stratum corneum. The TEM and PIXE studies never revealed the presence of particles within the cells of the living layers. These complementary techniques appear to be useful in providing clues to the percutaneous penetration.

065

Induction of Aquaporine 3 Expression and Filaggrin Degradation in Human Epidermis after Skin Barrier Disruption

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Aquaporin 3 (AQP-3) is a small transmembrane channel protein involved in water and glycerol transport in the epidermis. Filaggrin (FG) is an histidine-rich protein triggering microfilament compaction inside epidermal cells, keratinocyte differentiation into corneocytes, and providing, after hydrolysis, the natural moisturizing factor (NMF). Epidermis is a stratified, keratinised and cornified epithelium that forms the main barrier to body water loss. AQP-3 expression level has been previously shown to be coordinated with other epidermal proteins such as claudine-1, a tight junction protein, and with CD44, the receptor for hyaluronate, suggesting a tight control of water homeostasis inside epidermis. The present study investigates from a kinetic point of view, the effect of a superficial skin barrier disruption on both AQP-3 and FG expression. Barrier disruption was performed on human skin biopsies maintained at the air-liquid interface using serial tape stripping ($n = 10$) or lipid removal with a mixture of (ether/acetone) 1/1 v/v. AQP-3 and FG were immunostained on (7 μm) frozen skin sections and their expression measured for up to 4 days. The results showed that keratinocytes of control non-stripped skin expressed AQP-3 from the basal to the most superficial living cell layers, mainly in their plasma membrane, since FG was present only in the cytoplasmic compartment of granular cells. After barrier disruption, we observed a strong short term (3h and 24 h) decrease of FG expression. FG was then restored to a normal level at the basis of the stratum corneum after 24 h. In the same time, an important increase of AQP-3 expression was noted in the cytoplasmic compartment of the keratinocytes, mainly in basal cells, then, after 4 days, in their plasma membrane and in the entire epidermis. These results show that human epidermis responds to stratum corneum barrier disruption by inducing a coordinated FG degradation and AQP-3 biosynthesis and that a rapid restoration of NMF together with an important aquaporin-mediated glycerol and water flux are required to generate a rich moisturizing environment allowing barrier reconstruction by epidermal cells.

066 [Oral 053]

Regulation of NHE1 Expression Through Barrier Requirements

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We previously demonstrated the functional role of the Sodium-Hydrogen antiporter 1 (NHE1) for epidermal barrier homeostasis and repair. Fluorescence lifetime imaging (FLIM) visualized its effects on Stratum corneum (SC) pH directly, while electron microscopy revealed structural and functional consequences via SC pH. Subsequently, we showed how changes in NHE1 expression correlate to postnatal acidification of newborn SC. We found that during the immediate postnatal period NHE1 expression is reduced to presumably steady-state levels necessary after birth.

As NHE1 has been shown to be a housekeeping gene regulating intracellular pH in most tissues, a role it also has in epidermis with the notable addition of contributing to, if not being the main source of, acidity for the SC. We here investigated whether barrier requirements could alter expression levels despite the housekeeping/steady state expression to be expected in epidermis.

Following tape stripping, we observed a steady increase in suprabasal expression for up to 48 hrs, becoming detectable by immunohistochemistry as early as 6 hrs post-tape-stripping.

As transepidermal water loss (TEWL) is the single most important signal regulating barrier repair through lipid secretion and processing, we studied whether a dry or humid environment could affect NHE1 expression and localization. We found that humidity up-regulates suprabasal expression, while it is down-regulated in mice exposed to a dry environment. Similarly, fetal rat explants *in vitro* demonstrate that the up-regulation of NHE1 following exposure of the explant to the air-medium interface is further enhanced by occlusion.

In addition, numerous prior studies have shown that barrier repair initiates immediately following the insult and that by TEWL approximately 50% of experimentally induced damage is repaired within the first 5 hrs. This effect can be delayed, but not blocked by external pH-buffering, while pharmacologic blockade of NHE1 further delays barrier recovery.

As the observed changes in NHE1 expression are late events relative to barrier repair, or occur within the time of several days at the corresponding conditions, we conclude that NHE1 expression changes based on tissue turnover or chronic effects that affect SC pH. More immediate metabolic requirements, e.g. the rapid initial recovery in tape-stripping experiments, may be better and faster addressed by increased activity of the antiporter, a parameter which to date cannot be studied directly.

067

PA 102, a Fatty Acid Regulator of Epidermal Terminal Differentiation: Examination of Biological Mechanisms

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In mammals, epidermal differentiation and barrier homeostasis are critical components of skin barrier function. Both are coordinately regulated and the responses that occured after perturbations, used various operative signalling pathways. Previously, we showed that the fatty acid PA 102 profoundly influenced both morphological and biochemical aspects of epidermal differentiation *in vitro* : profilaggrin and filaggrin like most other differentiation markers of keratinocytes were up-regulated. Profilaggrin is an abundant component of keratohyalin granules and form the precursor of filaggrin, the keratin-associated protein of the stratum corneum.

Here we studied the effects of topically applied PA 102 in an *in vivo* model of barrier function disruption following a diet with essential fatty acid (EFA) deprivation, using histological and ultrastructural analysis of epithelium. Morphologically, PA 102 supplementation enhanced the normal wavy pattern of the stratum corneum and the number of keratohyalin granules found in epithelial granular layer. Histological data from cutaneous epithelium showed that PA 102 could decrease the mitotic cell number, limit the inflammatory infiltrate and regulate the epidermal differentiation with a marked effect on the filaggrin expression.

Recently expression of PPARs transcription factors in keratinocytes were demonstrated and ligands were found to enhance epidermal maturation. So we experimented PPAR-γ activation by PA 102 in HaCat keratinocytes and our data showed here that PA 102 activated the transcription factor in a dose-dependant manner.

In conclusion, in this *in vivo* model of a EFA-deprivated diet, inducing barrier disfunction with generation of cutaneous inflammation and epidermal hyperplasia, topical applying of PA 102 could restore skin barrier function through PPARs activation. By supporting physiological mechanisms to maintain a healthy skin barrier, PA 102 could be used for the treatment of dry skins associated with aged, atopic and psoriasis conditions.

068

Less Permeation Of Pimecrolimus Through Normal, Inflamed Or Corticosteroid Pre-Treated Porcine Skin As Compared To Tacrolimus

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The barrier function of the skin plays a pivotal role in the percutaneous absorption of epicutaneously applied drugs. Various inflammatory conditions or iatrogenic interventions are associated with disturbed barrier function and, therefore, can result in increased skin permeability and consequent enhanced systemic exposure to topically applied drugs. In a side-by-side comparison we evaluated the penetration and permeation of pimecrolimus and tacrolimus in normal, inflamed, and corticosteroid (CS) pre-treated porcine skin. Inflammation was induced locally by a 48-hr occlusive application of 5% sodium lauryl sulfate (SLS). CS pre-treatment was performed with hydrocortisone (1%), mometasone furoate (0.1%), or clobetasol-17-butyrate (0.01%), applied twice daily for 5 days prior to dissection of skin samples. Inflamed, CS pre-treated and contra-lateral normal pig skin samples were then used in Franz-type diffusion chambers *in vitro*. Pimecrolimus and tacrolimus were applied epicutaneously either as solutions (at 1.0% in propylene glycol/oleyl alcohol 9:1), or as the marketed formulations (Elidel 1% cream, Protopic 0.1% ointment). The latter were used in studies with CS pre-treated skin. Neither inflammation nor pre-treatment with CS significantly enhanced the levels of the two drugs in the skin (i.e. the penetration into the epidermis and upper dermis). SLS–induced inflammation, however, was associated with immediate passage of the compounds through the skin, as opposed to a latency period of approx. 17 hrs in normal skin; also, a significantly higher permeation rate through skin was observed (by factors up to 6.2). The permeation rate of pimecrolimus through irritated skin was similar to the rate of tacrolimus through normal skin (173 *vs.* 166 ng/ml/hr). With skin that was pre-treated with CS, permeation was increased by factors of 3.6 (pimecrolimus) and 1.7 (tacrolimus) as compared to normal untreated skin. These factors were independent of the potency of the CS used. The permeation of tacrolimus was 5.9 times higher through hydrocortisone pre-treated skin, 7.1 × higher through mometasone pre-treated skin and 3.5 × higher through clobetasol pre-treated skin than that of pimecrolimus. Since low permeation *in vitro* predicts low plasma levels and thus low systemic drug exposure *in vivo* after topical application to patients these findings appear be of clinical importance.

069

Skin Roughness Induces and Enhances Wrinkle Formation

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Fine wrinkles often increase in number and depth during periods when skin is rough. In order to explore the mechanism involved, we experimentally induced rough facial skin in 8 healthy male volunteers by washing the skin with soap 5 times per day for 1 week. Transepidermal water loss (TEWL), water content in the cornified layer and skin elasticity were evaluated, and skin surface textures and wrinkle formation were analyzed by video microscopy and on photographs of skin negative replicas taken on days 3, 5, 7 and 14 (1 week after last washing). The water content in the cornified layer decreased gradually from day 3 and showed the lowest value at day 7 after washing. Conversely, TEWL increased from day 3 and peaked at day 7. Both had recovered by day 14. The skin elasticity decreased in terms of Uf values (corresponding to final distension, which is related to skin extension) measured on a Cutemeter, from day 3 through day 14. As regards skin texture, rough patterns were observed from day 3 through day 14, and crow's-feet (wrinkles at the outer corners of the eyes) and wrinkles at the lower eyelid became deeper from day 5 through day 14 in most volunteers.

We conclude that the repeated washing with soap caused epidermal permeability barrier disruption and dryness of the cornified layer (typical skin roughness) in facial skin, leading to a decrease of skin elasticity, and these cutaneous changes appear to induce and enhance wrinkle formation.

070

Characterisation of Axillary Skin Surface pH and its Changes after Washing

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The skin surface pH is an important parameter of skin physiology and influences various other factors such as composition of stratum corneum lipids, hydration, barrier function and growths of microorganisms. While data on skin surface pH on the forearm and certain other parts of the body are easy to find in the literature, only very few studies exist which concern pH values in the axilla. The aim of our study was to characterise the axillary *in vivo* pH in men and women and to evaluate the influence of washing.

After a standardized run-in period of 7 days, the *in vivo* skin surface pH was measured in 20 healthy volunteers in three distinct locations of each axilla using a calibrated CK pH Meter PH 900. The pH value was measured at baseline and up to 6 hours after a standardized washing procedure with tap water.

There were no significant differences of skin surface pH between the central, proximal and distal part of the arm pit or between the left and right axilla. However, there was a statistically significant difference in axillary skin pH between male and female probands on both sides with more acidic values in women compared to men. After washing with water, the mean axillary pH significantly decreased in women. In men, the axillary pH slightly increased after washing.

In summary, there is a gender difference in axillary skin surface pH, the exact pathogenetic factors of which remain to be revealed in future research. Washing with water further increased the difference between male and female pH values.

071

Human Facial Pore Area And Sebum Are Affected By The Menstrual Cycle

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Noticeable pores are one of the facial problems that concern women of various ages. We have investigated the physiological characteristics of the cheek skin of healthy women volunteers. The group with larger pores was found to have more parakeratotic corneocytes and higher transepidermal water loss (TEWL) values. This suggests that women with large facial pores may have immature corneocytes. The group with larger pores was also found to have larger amounts of sebum, especially unsaturated free fatty acids such as palmitoleic acid and oleic acid. However, little is known about the effects of seasonal change or the menstrual cycle on facial pores.

Sequential observations revealed that the area of pores and the amount of sebum remained comparatively constant throughout the year. Further, the effect of hormonal changes on female facial pores and sebum was precisely observed throughout the phases of the menstrual cycle. The area of pores of woman in the luteal phase was larger than that in any other phase. Increases in the number of parakeratotic corneocytes and in the amount of sebum were also seen in the luteal phase. These results suggest that fluctuations of sex hormone during the menstrual cycle might affect the condition of facial pores, sebum and the skin.

072 [Oral 009]

Implication of Collagen Type Vii In The Regulation of Motility and Invasion of Skin Keratinocytes

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Recessive dystrophic epidermolysis bullosa (RDEB) is an inherited skin blistering disorder caused by mutations in the gene coding for the collagen type VII (colVII), the major component of the anchoring fibrils. In RDEB, absent expression of colVII results in aggressive squamous cell carcinomas (SCC) associated with the unremitting healing of the skin lesions. Primary RDEB keratinocytes display altered migration and invasion properties ex vivo, which indicates a possible proneness to tumor progression. To explore the relationship between collagen type VII expression and the behavior of RDEB keratinocytes, we have analyzed RDEB cell cultures before and after transduction with a wild-type collagen type VII cDNA. Cell migration and invasion in haptotactic assays in Boyden chambers showed that RDEB keratinocytes display a 3-fold invasive capacity and a 2,5-fold hypermotility compared with normal keratinocytes. In contrast, the reverted r-RDEB cells behaved like the normal control. Expression of colVII in rRDEB cells or addition of exogenous collVII revert the hypermotility and invasion capacity of RDEB cells. The invasive potential of RDEB keratinocytes was neutralized by chemical inhibitors of the matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPa). Consistently, enzymatic assay showed a high MMP and uPA activity in RDEB keratinocytes that was reduced in the rRDEB counterparts. Western blot analysis using a panel of antibody specific to each MMP detected down regulation of MMP3, upregulation of MMP9 and overexpression of MMP1.

Our results show that collagen type VII plays a major role in keratinocyte migration with the involvement of specific MMPs including MMP1 and uPA.

073

***Trans*-splicing in GABEB Keratinocytes**

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Gene therapy in skin diseases is often restricted by the integration limits of commonly used viral and non-viral vectors in recessive diseases and the lack of promising strategies in dominant disorders. To overcome this problem the size of the therapeutic insertions can be reduced by a novel strategy defined as spliceosome-mediated RNA trans-splicing (SMaRT), which provides intron-specific gene-correction at the pre-RNA level by replacing mutant exons. Mutations in the COL17A1 gene, coding for a 6,5kB mRNA, are involved in the pathogenesis of Generalized Atrophic Benign Epidermolysis Bullosa (GABEB; nonHerlitz junctional EB). In previous experiments a β-galactosidase (β-gal) double transfection *trans*-splicing assay in 293T cells was evaluated, which was established using intron 51 of the COL17A1 gene as the target for *trans*-splicing. We observed expression of the β-gal protein only in cotransfected cells. Sequence analysis showed accurate splicing junctions (Dallinger *et al*, Exp. Dermatol 2003 Feb;12(1): 37–46). In this study we provide information about the feasibility of *trans*-splicing in the mutated endogenous gene responsible for GABEB. Transfection of a pre-trans-splicing molecule (PTM) harbouring exons 52–56 of the COL17A1 gene demonstrated replacement of the complete 3'-end of the COL17A1 gene in almost 50% of analysed clones. Correct *trans*-splicing and the correction of the 4003delTC mutation could also be verified by sequencing. Furthermore, cultured GABEB keratinocytes transfected with exon 52–56 PTM's resulted in the expression of type 17 collagen protein on the surface of the GABEB cell line shown by immunofluorescence. To test whether SMaRT produced mature COL17A1 protein, western blotting was performed with lysates of PTM-transfected cells. A 97kD band was detected by antibody LAD-1 recognizing the extracellular portion of type XVII collagen. These observations suggest that SMaRT may represent a feasible tool for reprogramming sequences of KC specific transcripts.

074

Detection of Human Serum Autoantibodies Against BP180 NC16a Domain by Surface Plasmon Resonance

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Introduction: Among immunological assays for the detection of autoantibodies in autoimmune bullous diseases Surface Plasmon Resonance (SPR) appears to be a novel useful biosensor instrument that is efficient, sensitive, accurate and reproducible. Intrinsec specificity of SPR is to measure binding kinetics of antigen-antibody interaction. Mapping of affinity constants (association or dissociation constants) can be performed simultaneously for three epitopes. Most of studies based on antigen-antibody interaction by SPR used monoclonal antibodies immobilized on chips. On the opposite, studies using antigen immobilization for the detection of polyclonal antibodies in human serum are rare.

Objective. To evaluate efficacy, sensitivity and reproducibility of SPR for detection of serum human antiBP180 NC16a antibodies.

Materials and methods. Twenty sera containing antibodies against BP180 were selected. They were previously characterized by immunoblot on epidermal extract (11 bullous pemphigoid, 2 pemphigoid gestationis, 7 cicatricial pemphigoid). Indirect immunofluorescence on salt split skin was performed for all sera. Indirect or direct immunoelectron microscopy were performed for most of them. Control sera provided from 2 healthy subjects, from 1 patient with pemphigus vulgaris and 1 patient with superficial pemphigus. A GST-recombinant NC16a peptide was used. A ELISA assay with the GST-recombinant NC16a peptide was finalized according to previously published techniques. Detection of antibodies by SPR (Biacore®) was performed with the GST-recombinant NC16a peptide and with GST alone as control.

Results. Detection of serum antibodies against BP180 NC16a by SPR was possible and reproducible (variation of 12%). High level of proteins and antibodies in human serum did not interfere until 1/50 dilution. Sensitivity of SPR was quite lower than sensitivity of ELISA. Therefore it was enhanced with the introduction of secondary antibodies (human anti-IgG). Elisa and SPR results were concordant for bullous pemphigoid patients. Curves of sera were unusual for pemphigoid gestationis. Sera of cicatricial pemphigoid, lowly positives by ELISA were systematically negatives by SPR.

Discussion. Detection of serum antibodies against BP180 NC16a is possible by SPR. SPR is a fast (10 minutes) and reproducible technique. Optimizing parameters of SPR (concentration of antigen on the chip, flow rate), SPR should be as sensitive as ELISA. Therefore SPR is especially useful to measure binding affinity constants. Using this ability, SPR allows an original approach to study polyclonal immune response in autoimmune bullous diseases.

075

Induction of Dermal-Epidermal Separation In Mice by Passive Transfer of Antibodies to Type Vii Collagen

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Autoimmune conditions are characterized by autoreactive T cells and/or autoantibodies that can transfer the disease. Epidermolysis bullosa acquisita is a subepidermal blistering disease associated with tissue-bound and circulating autoantibodies to type VII collagen, a major constituent of the dermal-epidermal junction. Previous attempts to transfer the disease to mice by injection of patient autoantibodies have been unsuccessful, possibly due to their reduced reactivity with murine skin. To study the pathogenic relevance of antibodies to type VII collagen, we generated a recombinant form of murine type VII collagen which was used to immunize rabbits. Antibodies from these rabbits bound to the lamina densa of murine skin and immunoblotted type VII collagen. When purified rabbit IgG to type VII collagen was passively transferred into adult BALB/$c^{nu/nu}$ mice (n = 12), all animals, in contrast to control mice (n = 4), developed widespread skin blisters. By direct immunofluorescence microscopy, tissue-bound rabbit IgG was detected that fixed murine C3 to the dermal-epidermal junction. Histopathological examination of mouse lesional skin revealed dermal-epidermal separation accompanied by an inflammatory infiltrate dominated by neutrophils. By electron microscopy, the cleavage plane was shown to localize to the sublamina densa. In the serum of injected mice, titers of rabbit IgG to type VII collagen correlated with the extent of skin disease. This novel animal model duplicates the key findings in patients with epidermolysis bullosa acquisita. It should greatly facilitate the further dissection of the pathogenesis of this disease and the development of new therapeutic strategies.

076

Laminin 5-specific IgG Autoantibodies in Mucous Membrane and Bullous Pemphigoid

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Mucous membrane pemphigoid (MMP) is a chronic autoimmune bullous disease which mainly affects mucous membranes leading to scarring of the affected tissue. Patients with MMP have serum antibodies (ab) that preferentially recognize two components of the dermoepidermal basement membrane zone (BMZ), BP180 and laminin 5 (LN5). Since the detection of serum auto-ab is critical for establishing the diagnosis of MMP, we sought to develop a novel ELISA with native LN5 derived from squamous carcinoma cell (SCC-25)-conditioned medium. A total of 24 MMP, 72 BP and 51 control sera were analysed for LN5-specific auto-ab. By ELISA, 18/24 (75%) MMP and 29/72 (40.3%) BP sera contained IgG reactive with LN5. Thus, the sensitivity and specificity of LN5 ELISA were for MMP 75% (95% CI: 53.3–90.2%) and 84,3% (95% CI: 71.4–93.0%) respectively, and for BP 40,3% (95% CI: 28.9–52.5%) and 88,2% (95% CI: 76.1–95.6%) respectively. In the MMP patients, the titers of LN5-reactive IgG correlated well with clinical disease severity. As an example, in a patient with severe MMP, the serum IgG titers were closely related to clinical disease activity. In contrast, there was not such a relationship between LN5-reactive IgG and clinical severity in the LN5-reactive patients with BP. Furthermore, LN5 IgG responsiveness of the MMP and BP sera was not significantly associated with IgG reactivity against other components of the BMZ, such as BP180 and/or BP230. The established LN5 ELISA is highly sensitive and specific and holds great promise as a novel diagnostic and prognostic parameter in MMP.

077

Novel *COL17A1* Mutations In Junctional Epidermolysis Bullosa Patients From The Netherlands

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Mutations in the gene *COL17A1*, coding for type XVII collagen, cause non-Herlitz junctional epidermolysis bullosa (nH-JEB), although occasionally features of epidermolysis bullosa simplex (EBS) are observed. Here we give an overview of 10 epidermolysis bullosa patients from the Netherlands with mutations in *COL17A1*. Immunofluorescence antigen mapping with monoclonals 1A8C and 1D1, specific for respectively the intracellular and extracellular domain, revealed absence of type XVII collagen in the 8 patients with a classic nH-JEB phenotype. In contrast, reduced expression of type XVII collagen was observed in the 2 patients (1877-2A > C/3432delT and 2356C > T/3432delT) with a mild form of the disease. The splice-site mutation in intron 21 (1877-2A > C) generated different mRNA transcripts resulting in the deletion of exon 22, while in case of the 2356C > T mutation type XVII collagen was rescued by deleting the premature termination codon containing exon 30. Accordingly, in both cases a smaller type XVII collagen protein could be detected on immunoblot. Ultrastructural analysis demonstrated junctional cleavage through the lamina lucida in 9 patients. One child (1877-2A > C/3432delT), however, had an intra-epidermal ('pseudojunctional') cleavage level fitting EBS rather than nH-JEB. Furthermore, genomic DNA analysis identified in the patient group four novel deletions, all leading to a premature termination codon. A 2-year-old girl had the deletions 1365delC in exon 16 and 3600-3601delCT in exon 49. Another mutation, 1284delA in exon 15, was present in a 5-year-old boy, in addition to the known regional 3432delT mutation. The last deletion, 3236delC in exon 46, was found in a 45-year-old woman of consanguineous not-affected parents. Besides these new findings, DNA analysis also showed that the mutations 2342delG, 3432delT and 3781C > T were each occurring in three unrelated patients. Our data give further insight into genotype-phenotype correlation in patients with mutations in *COL17A1*.

078

Anti-plectin Autoantibodies in Pemphigoid

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Hemidesmosomal protein components may become autoimmune targets in pemphigoid diseases. Well-known auto-antigens are the intracellular plaque protein BP230, the transmembrane component BP180 and its shed extracellular domain LAD-1. Here we show that plectin, another hemidesmosomal intracellular plaque protein, may also become an auto-antigen. This study had three goals: to establish the existence of anti-plectin antibodies in pemphigoid patients, to determine the incidence of these antibodies, and to map the distribution of epitopes over the plectin molecule. By routine immunoblotting analysis of patient sera, we found among 283 cases 12 patients with an immunoblot staining pattern identical to that obtained with anti-plectin monoclonal HD121. When these serum antibodies were affinity-purified by eluting them from the blot, they bound to normal human skin in a pattern typical for plectin: along the epidermal basement membrane, in keratinocytes in the epidermis and in myocytes in the musculus erector pili consistent with the expression of plectin in epithelial and muscular tissue. The affinity-purified antibodies did not bind to plectin-deficient skin of a patient with epidermolysis bullosa simplex with muscular dystrophy, supporting their pure anti-plectin nature. To map the epitopes on the plectin molecule, recombinant constructs of different plectin domains were expressed in COS-7 cells and used for immunoblotting with anti-plectin patient sera. The central coiled-coil rod domain appeared an immunodominant hotspot as 7 out of 9 (78%) sera with anti-plectin antibodies reacted with it. Most patients had accompanying antibodies against other pemphigoid antigens as BP230 and BP180, making it difficult to disclose the pathogenicity and the clinical manifestations of anti-plectin auto-antibodies. However, one patient had an IgA and IgG response to plectin only, which manifested as a mild serpenginous vesicular skin eruption resembling linear IgA dermatosis. Plectin is the fourth auto-antigen in pemphigoid, of which humoral auto-immune reactions may cause the subepidermal autoimmune bullous disease.

079

Autoantibodies to Human α6 Integrin in Patients with Bullous Pemphigoid

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Bullous pemphigoid (BP), an IgG-mediated autoimmune blistering disease, is characterized immunologically by tissue-bound and circulating autoantibodies targeting the hemidesmosomal cytoplasmic plaque protein BP230 and the transmembrane protein BP180. The α6β4 heterodimer is an integrin family of adhesion receptors, which mediate basal keratinocytes to extracellular matrix interactions. It was earlier demonstrated that α6 integrin is a BP180 binding partner in the hemidesmosomal multimolecular complex and that this connection is essential for the function of hemidesmosomes. Recent evidence suggested a pathophysiological role for autoantibodies against α6 integrin in the subepidermal blister formation of oral pemphigoid. The objective of our study was to investigate the presence of anti-α6 integrin antibodies in patients with BP. The autoantibody profiles of 30 patients with BP, 5 patients with pemphigus vulgaris and 10 healthy persons were identified. With the use of PeptideStructure and PlotStructure software, antigenic epitopes for α6 integrin were predicted. One intracellular (NKDA 1045-1073) and three extracellular antigenic epitopes (TPAC 477–489; SVLP 585–601; and SPDA 681–695) were chosen. The coding sequences of these antigenic epitopes were chemically synthetized and inserted as homo- or hetero-oligomeric forms into fusion-expression plasmids (PGEX-4T, Pharmacia). Fusion products were expressed and purified from *E. coli* cells by affinity chromatography. Sera were tested for α6 integrin autoantibodies with the fusion recombinant proteins in an ELISA system. 28% of the BP sera reacted with the construct containing the intracellular epitope, but positive reactions (32 and 36%) were demonstrated more frequently against the recombinant proteins containing the extracellular antigenic peptides of α6 integrin which are close to the transmembrane region. Altogether, 52% of the patients with BP displayed circulating antibodies against at least one recombinant protein. The healthy persons and the patients with pemphigus vulgaris did not exhibit immune reactivity nor recombinant constructs. As the BP patients with autoantibodies against α6 integrin did not reveal mucosal involvement of their disease, the presence of α6 integrin autoantibodies might be a secondary autoimmune event, a consequence of epitope spreading. However, the pathogenic role of anti-α6 integrin autoantibodies in BP demands further investigations.

080

Cytoskeletal Protein Network Environment of Bullous Pemphigoid Antigen 2 (BPAG2) Validated by a New Computational Protein Interaction Methodology

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BPAG2 is a transmembranous hemidesmosomal collagen (Col17A1) associated with human diseases such as generalized atrophic benign and junctional epidermolysis bullosa. The main cellular function of BPAG2 is the adhesion of keratinocytes to the basement membrane and the maintenance of the structural integrity of the cell. An exhaustive yeast two-hybrid screen with BPAG2 gene as the bait revealed numerous putative novel protein interaction partners. We used a human keratinocyte prey library and identified twenty-nine different annotated and seventeen so far unknown gene sequences as presumptive interaction partners. The majority of these interaction partners are structural components of the cytoskeleton. A highly concentrated actin related environment for BPAG2 was noticed. Since in eukaryotic cells a network of proteins which constitute microtubules, actin filaments and intermediate filaments is responsible for structural integrity, cell motility and adhesion, it is not surprising to identify a main core of proteins related to the cytoskeleton. Shot gun experiments like the yeast two-hybrid system often deliver a wealth of biological information which have to be put into a correct perspective and eliminated for false positives. Therefore protein sequence homology based computational approach was designed and used for a rapid selection and validation of experimentally collected protein interaction data. These experiments constitute the initial series on the pathway to the keratinocyte proteome.

081

Performances of the SCORTEN During the First Five Days of Hospitalisation

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Introduction: The SCORTEN calculated within 24 hours of admission, is a severity-of-illness score validated for toxic epidermal necrolysis (TEN) and Stevens-Johnson Syndrome (SJS). Our purpose was to assess the performances of successive SCORTENs and to determine the influence of admission delay.

Methods. Medical charts of patients admitted for TEN, SJS or TEN/SJS overlap syndrome in our dermatology intensive care unit between 1993 and 2003, and not included in a therapeutic trial, were reviewed. The SCORTEN parameters (age $\geqslant$40 years, cancer, epidermal detachment $\geqslant$10%, tachycardia $\geqslant$120/mn, serum urea $>$10 mmol/l, serum glucose $>$14 mmol/l, serum bicarbonate $<$20 mmol/l) collected from Day 1 to Day 5, as well as the SCORTENs, were compared between deceased patients (n = 28,19.4%) and survivors (n = 116). The performances of the score, calibration (Hosmer-Lemeshow test) and discriminatory power (ROC curves) were assessed from D1 to D5. Admission delays (D1–Day of first cutaneous or mucous symptoms) were compared between these two populations, and the D1 SCORTEN was adjusted for this delay.

Results. Data of 144 patients (74 men/70 women), aged 46.8y ($\pm$ 19.7) were analysed. All seven SCORTEN parameters, on whichever day collected, were associated with a higher mortality rate. The SCORTEN rose slightly during hospitalisation with a significant difference between D1-D2 (0.006) and between D1-D4 (0.001). Predictive and discriminatory values of the SCORTEN were good from D1 to D5, with few differences between the expected and observed numbers of death, and with areas under ROC curves (AUC) all above 80%.

The admission delay did not differ between deceased patients and survivors: 86% of the deceased and 80% of the survivors had a delay $\leqslant$7 days. Delay adjusted SCORTEN was close to the crude SCORTEN.

Discussion. We established an additional validation of the SCORTEN (2/3 of the patients were included in the initial study) and demonstrated that the SCORTEN performances are not altered during the first 5 days of hospitalisation. The SCORTEN is a good predictor even for late deaths. The admission delay had no significant role on prognosis nor on SCORTEN's value. This could be explained by early pre-admission deaths, and moreover by a later admission of less severe cases.

082

Coexistence of IgA Antibodies to Desmogleins 1 and 3 in Pemphigus Vulgaris, Pemphigus Foliaceus and Paraneoplastic Pemphigus

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Pemphigus is a bullous mucocutaneous autoimmune disease characterized by IgG autoantibodies to desmoglein 1 (Dsg1) and/or desmoglein 3 (Dsg3). Occasionally direct IF of pemphigus skin reveals IgA depositions with an intra-epidermal intercellular pattern in addition to IgG. In this study we investigated if pemphigus patients also generate IgA antibodies to Dsg1 and Dsg3. Therefore we tested 96 pemphigus patients and 28 bullous pemphigoid controls by IgA-ELISA to the recombinant extracellular domains of Dsg1 and Dsg3. Pemphigus patients were selected on clinical grounds and positive IgG-ELISA index values for Dsg1 and/or Dsg3. The 96 pemphigus patients were divided as follows: 33 had pemphigus foliaceus (PF) with IgG to Dsg1, 28 had pemphigus vulgaris (PV) with IgG to Dsg3, 28 had PV with IgG to both Dsg3 and Dsg1, and 7 had paraneoplastic pemphigus (PNP). The results were as follows: IgA antibodies to Dsg1 were found in 14 (42%) of the PF patients, in 10 (18%) of the PV patients, and in none of the PNP patients. IgA antibodies to Dsg3 were found in 1 (3%) of the PF patients, in 24 (43%) of the PV patients, and in 3 (43%) of the PNP patients. In most cases the specificity of the IgA followed the specificity of the IgG, although in a minor number of cases IgA was present against the desmoglein not recognized by IgG. This study shows that in a considerable number of supposedly IgG-mediated pemphigus patients also IgA to Dsg1 and Dsg3 is present. In order to fully understand the pathogenesis of pemphigus it will be necessary to investigate if this IgA contributes to the pathological mechanism of blister formation in pemphigus.

083

Laser Scanning Confocal Microscopy Study in a Case of Atypical Pemphigoid with Antibody Against 200kd protein

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We present a 54-year-old female with tense blisters forming targetoid-like lesions on the flexural surfaces of the upper limbs, inner tighs, wrists and the presence of *in vivo* bound and circulating IgG anti basement membrane zone antibody (aBMZAb) disclosed by immunofluorescence (IF) study. Skin lesions poorly responded to prednisone 60 mg/daily for 2 months. While on steroid treatment she developed erythema gyratum repens-like lesions, however laboratory investigations did not reveal internal malignancy. The aim of the study was to characterize the target antigen, its ultrastructural localization and the localization of *in vivo* bound IgG at the BMZ. Laser scanning confocal microscopic (LSCM) study was performed to compare the localization of *in vivo* bound IgG to the localization of various BMZ markers. Immunoglobulins were visualized by labeling with FITC-conjugated goat anti-human IgG antibodies, whereas basement membrane zone markers were labeled with Cy5-conjugated anti-mouse antibodies. IF images were overlaid by an image processing system integrated in the LSCM and photographed. Confocal microscopic examination disclosed the presence of *in vivo* bound IgG above collagen IV and on the level of laminin-5, which corresponded to their ultrastructural localization at the lamina lucida-lamina densa border. Serum study on salt split skin showed reactivity with the floor of the blister. LSCM study disclosed the localization of target antigen above the collagen type IV, at the level of laminin-5. Western immunoblot study performed on dermal extract showed reactivity of patient's serum with 200 kDa protein. IF study performed on the skin derived from generalized epidermolysis bullosa dystrophica showed lack of expression of 200kD antigen in comparison to normal human skin, which may suggest a functional relationship between collagen VII and protein 200kD at the BMZ. Our LSCM study showed that *in vivo* bound aBMZ antibody and target antigen 200kD are localized at the lamina lucida-lamina densa border.

084

Multiforme-like Adverse Skin Reactions in Association with the Use of Bextra® (valdecoxib)

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Bextra® is a new COX-2 inhibitor with valdecoxib as its pharmacological component. Like sulfonamide antimicrobials valdecoxib contains a sulfonamide structure. Sulfonamide antimicrobials (especially co-trimoxazole) are known for inducing severe skin reactions like Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). Since March 2003 Bextra® has been introduced into the EU pharmacological market and since May 2003 it can be obtained in Germany.

Between July and October 2003 the German registry of severe skin reactions has evaluated 4 suspected cases of SJS after the use of valdecoxib. These patients developed moderate to severe adverse skin reactions 5 to 11 days after daily intake of Bextra®. Clinically they all showed a generalized erythema in the beginning and during the course a maculopapular exanthema with a tendency to develop target-like lesions (3/4) and blisters (2/4), emphasizing the trunk. In addition some patients had fever up to 39 degree Celsius (3/4), mild dyspnea (2/4), facial edema (4/4), pruritus (2/4) as well as mild mucosal involvement (3/4).

These skin reactions do not fit into the consensus definition of SJS and TEN. Moreover, it seems that the pattern of the reaction described after the intake of COX-2 inhibitors is that of specific multiforme-like drug reactions. In this evaluation we focused on valdecoxib, but very similar drug reactions are also known after the use of celecoxib, another COX-2 inhibitor introduced into the pharmacological market in 2000.

Since drug reactions to Bextra® have been observed already shortly after its introduction to the market and similar reactions have been described for other COX-2 inhibitors, the drug seems to have a potential to induce cutaneous adverse reactions.

Taking into account other published case reports, COX-2-inhibitors seem to induce class-specific multiforme-like skin reactions and perhaps also more severe adverse reactions like SJS and TEN. Therefore, COX-2-inhibitors should be carefully monitored in terms of cutaneous adverse reactions.

085

Expression of Matrilysin-1 (MMP-7) in Cutaneous Squamous Cell Carcinoma in Recessive Dystrophic Epidermolysis Bullosa

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Cutaneous squamous cell carcinoma (SCC) occurs frequently in chronic ulcers in patients with recessive dystrophic epidermolysis bullosa (RDEB). Although the cutaneous SCCs in RDEB are in general histologically well differentiated they are characterized by rapid invasion and development of metastases resulting in poor prognosis. In this study, we wanted to elucidate the role of extracellular matrix degrading enzyme matrilysin-1 (matrix metalloproteinase-7; MMP-7), in these carcinomas.

Formalin-fixed, paraffin embedded samples were obtained from 8 cutaneous SCCs and 2 lymph node metastases from 6 RDEB patients (ages between 12 to 44 years, 2 Hallopeau Siemens RDEBs, 2 non Hallopeau Siemens RDEBs, 2 not specifically defined RDEBs). Tumor sections were stained by immunohistochemistry using mouse monoclonal antibodies against MMP-7 and cell surface protein syndecan-1, a substrate for MMP-7. Negative control stainings were performed without primary antibody.

Of the 8 SCCs examined, 7 tumors were histologically well differentiated and 1 tumor was moderately differentiated. In tissue sections from all 8 SCC cytoplasmic staining for MMP-7 was seen in tumor cells but not in stromal cells. MMP-7 expression was also detected in tumor cells in both lymph node metastases examined. MMP-7 was also expressed by exocrine epithelial cells in sweat glands where as no staining was noted in normal epidermis or in negative control stainings. Abundant expression of syndecan-1 was noted in the cell surface of keratinocytes in stratum spinosum of normal epidermis. Syndecan-1 expression in tumor cells in SCCs was focal, irregular and clearly diminished, as compared to normal epidermal keratinocytes.

These results show that the expression of MMP-7 is specifically induced in malignantly transformed keratinocytes in this aggressive subset of cutaneous SCCs as well as in their lymph node metastases suggesting a role for MMP-7 in early SCC development and metasis. These results also indentify MMP-7 as a potential therapeutic target to inhibit growth and invasion of SCCs in patients with RDEB.

086 [Oral 064]

Processing of the Short Arm of the Laminin gamma2 Chain Modulates Adhesive, Migratory and Growth Capacity of Laminin 5

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Laminin 5 is the major adhesive ligand of epithelial cells which has been also involved in the regulation of various cellular processes including migration, proliferation and apoptosis. Extracellular laminin 5 harboring two distinct proteolytical forms of the gamma2 chain is generated by cleavage in the NH(2)-terminal short arm of the gamma2 polypeptide that reduces the size of gamma2 chain from 155 to 105 kDa or 80 kDa. The 105 and 80 kDa polypeptides are found respectively in the basement membrane under physiological conditions and in remodeling tissue or carcinoma. However, the specific function of these molecules remains obscure. To address the biological role of the gamma2 chain processing, both *in vitro* and *in vivo*, we expressed the unprocessed and processed forms of the gamma2 chain into primary gamma2-null keratinocytes and generated a knock-in mice that exclusively expressed laminin 5 molecules harboring an unprocessed gamma2 chain by mutation in the proteolytic cleavage site. Analysis of the transgenic animal shows accumulation of laminin 5 and other components of the ECM in the basement membrane which correlates with a lamina densa thickening. *In vitro* studies confirmed that the laminin 5 with the unprocessed gamma2 form is actively integrated in the extracellular matrix, supports cell adhesion, activates epithelial migration but inhibits cell proliferation. Interestingly, expression of the 80 kDa gamma2-null keratinocytes failed to revert the adhesion defect of the recipient cells but activates keratinocyte proliferation and migration, strenghtening the role of this molecule in tumor progression. In conclusion, our results clarify the cellular fonction of the gamma2 chain processing and elucidates the specific role of the different extracellular forms of the laminin 5 in a physiological and pathological context.

087 [Oral 003]

The Laminin 5-binding Domain of BP180/collagen XVII is Important for Structural Integrity and Adhesive Functions

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Collagen XVII, a transmembrane component of the hemidesmosomes, mediates adhesion of the epidermis to the underlying basement membrane by binding to laminin 5. Genetic defects of collagen XVII are associated with epidermal detachment in junctional epidermolysis bullosa (JEB). Here we analyzed the biologic consequences of a spontaneous deletion of the distal C-terminus of collagen XVII in a JEB patient. The underlying mutation was a homozygous duplication of four nucleotides in exon 54 of the COL17A1 gene. It resulted in a frame shift, an adjacent nonsense sequence of 18 amino acids, a premature termination codon and elimination of 43 most C-terminal amino acids of the collagen XVII ectodomain, including the laminin 5 binding domain. The mutation led to expression of a truncated molecule, albeit at a somewhat lower level. Immunofluorescence staining with antibodies to N-terminus of collagen XVII resulted in a positive signal in JEB skin, but an antibody against the distal C-terminus remained negative, indicating that a deleted molecule was stable *in situ*. Immunoblots revealed both truncated collagen XVII forms in JEB keratinocyte cultures, the transmembrane form and the shed ectodomain. Analysis of the thermal stability by limited trypsin digestion showed that the helix-to-coil transition temperature was significantly lower for the truncated collagen XVII than for wild type controls, indicating abnormal folding of the mutant molecule. Analysis of keratinocyte migration by cell scattering assays showed the JEB cells to be more motile than normal keratinocytes, but in an undirected manner. Comparable motility characteristics have been observed before with collagen XVII deficient keratinocytes. These findings demonstrate that the deletion of the laminin 5-binding domain of collagen XVII causes abnormal folding, inhibits physiologic ligand binding, and possibly increases the susceptibility of the ectodomain to proteolytic degradation. The findings underline the role of the distal ectodomain of collagen XVII in binding to laminin 5, epidermal adhesion and etiopathogenesis of JEB.

088

Morphogenesis of Dermo-epidermal Junction in a Model of Reconstructed Skin: Beneficial Effects of Vitamin C

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In skin, cohesion between dermis and epidermis is ensured by the dermal-epidermal junction (DEJ). It is also required for normal control of epidermal growth and differentiation. Reconstructed skin comprising both a fully differentiated epidermis and a dermal equivalent with human fibroblasts is a model of choice to study DEJ.

In this study we showed that addition of vitamin C optimised the DEJ formation in an *in vitro* human reconstructed skin model leading to DEJ structure closer to that of normal human skin. In comparison with classical culture conditions, vitamin C treatment led to a better organization and architecture of basal keratinocytes, an increase in fibroblast number and a faster formation of DEJ. Actually, by imunolabeling, we observed that vitamin C accelerates deposition at the DEJ of several basement membrane proteins, like type IV and VII collagen, laminin-1, type I and III procollagens, Tenascin and Fibrillin. Mechanism of action of vitamin C was investigated by real time PCR on the sub-cited basement membrane markers, in fibroblasts and keratinocytes respectively. Our results showed that 1- gene modulation induced by vitamin C was dependent on the cellular type: the fibroblast for modulation of col I α1, col III α1, fibrillin and laminin γ1, the keratinocyte for col VII α1, col IV α2 and laminin β3; 2- vitamin C effects on DEJ passed in part through a transcriptional pathway for procollagen I and III, Fibrillin and for laminin γ1; whereas effects on the other DEJ markers appeared to happen at the traductional and/or post-traductional level. These data reinforced the knowledge on vitamin C effect on DEJ formation and bring new insights on the potential use of such molecule to improve or regenerate this zone.

089 [Oral 015]

Hair Cycle-Dependent Expression of a Novel Component of the Hair Follicle-Dermis Junction, Collagen XXII

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Collagen XXII is a novel member of the FACIT protein family (fibril associated collagens with interrupted triple helices), which was first identified by screening an EST database, subsequently expressed as a recombinant protein and characterized as an authentic tissue component and cell adhesion ligand. It has a uniquely restricted expression pattern at tissue junctions in the skin, muscle, heart and articular cartilage. In the skin it is localized in the basement membrane zone between the hair follicle and the dermis, but not below the interfollicular epidermis. In both human and murine skin, collagen XXII is located in the lower segment of the anagen hair follicle, between the outer root sheath keratinocytes and the myofibroblast layer surrounding the lower follicle. Immunoelectron microscopy demonstrated gold particles along the interdigitated basement membrane. Intriguingly, the expression of collagen XXII is hair cycle-dependent. During the first hair cycle of newborn mice and during synchronized hair cycles, the expression was strongest during anagen growth phase on day 1–12, then slowly diminished during the catagen phase and became negative in the telogen phase by day 18–22. *In vitro* collagen XXII is synthesized by HaCaT keratinocytes and smooth muscle cells, as assessed by RT-PCR analysis and immunofluorescence staining. This suggests that both the epithelial and mesenchymal tissue compartments contribute to collagen XXII synthesis in the skin. Taken together, the restricted expression in the transient regions of the hair follicle basement membrane and the absence during the involution and resting stages of hair cycles suggest that collagen XXII reflects and/or influences epithelial-mesenchymal interactions during hair follicle morphogenesis and cycling.

090 [Oral 013]

Collagenase-3 in Fibroblast-mediated Collagen Matrix Remodelling

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Collagenase-3 (MMP-13) is a matrix metalloproteinase (MMP), which is not present in normal adult human skin wounds but is expressed by fibroblasts in non-scarring adult gingival and fetal skin wounds. We have studied the role of MMP-13 and tissue inhibitors of MMPs (TIMPs) in fibroblast-mediated contraction and remodelling of collagen matrix, a process that takes place during acute wound healing in skin.

We have used adenovirus-mediated gene delivery of MMP-13, TIMP-1 and TIMP-2 to human adult skin fibroblasts and studied their contraction capacity and morphology in floating 3D collagen lattice.

Adenoviral expression of human MMP-13 increased the collagen contraction capacity of dermal fibroblasts by 60% when compared to control virus infected cells. The contraction was partly inhibited by adenoviral co-expression of TIMP-1 and TIMP-2. Addition of human recombinant MMP-13 also induced collagen contraction by dermal fibroblasts. Adenoviral expression of MMP-13 caused fibroblasts to form shorter but more complex actin-containing cell extensions than the control cells in 3D collagen. The results suggest that MMP-13 induces the reorganization of collagen by fibroblasts. The inhibitory effect of TIMP-1 and -2 on MMP-13 induced collagen contraction and the changes in actin cytoskeleton of fibroblasts expressing MMP-13 suggest that partial degradation of collagen and probably enhanced interaction between fibroblasts and collagen are involved in the process. We conclude that MMP-13 may also *in vivo* enhance wound contraction and even promote scarless healing of adult gingival and fetal skin wounds by enhancing matrix remodelling.

091 [Oral 008]

Tenascin-X: An organizer or stabilizer of the extracellular matrix?

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Tenascin-X (TNX) is a large 450 kDa extracellular matrix (ECM) protein. All members of the tenascin family have a similar domain structure consisting of multiple EGF-like and fibronectin III (FNIII) repeats and a C-terminal fibrinogen-like domain. TNX deficiency leads to a recessive form of Ehlers-Danlos syndrome (EDS), characterised by hyperextensible skin and joint hypermobility. In this study we have investigated skin biopsies of these patients to elucidate possible alterations in their connective tissue. In addition we have started to map possible interaction sites of TNX with other ECM components. We show that TNX deficient patients have abnormal elastic fibers and reduced collagen content in the dermis. The coarse elastic fibers of the reticular dermis appeared clumped and fragmented, while the fine elastic fibers of the papillary dermis are less branched and reduced in number and size compared to healthy individuals. At the ultrastructural level immature and irregularly shaped elastin fibers, some devoid of microfibrils, were observed. This indicates an important role for TNX in the organization or stabilization of the ECM. Using a solid-phase assay, we show that a 100 kDa C-terminal part of TNX, encompassing the last six FNIII repeats and the fibrinogen-like domain, binds to collagen type I and tropoelastin. Recombinantly expressed TNX fibrinogen-like domain shows binding to tropoelastin but fails to show binding to collagen type I. This suggests that the collagen binding site is located within the FNIII domains.

It remains to be investigated whether the interaction of TNX with other ECM molecules is involved in the initial assembly of ECM structures or serves as a factor in maintenance and turnover.

092

Novel Glycine Substitutions in the Largest Collagenous Domain Col15 Decrease the Thermal Stability of Collagen XVII

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Collagen XVII is a hemidesmosomal transmembrane component with a globular cytoplasmic domain and a large extracellular domain with interrupted collagenous subdomains. Mutations in COL17A1 gene lead to junctional epidermolysis bullosa, a heritable disorder with features of skin fragility, abnormal hemidesmosomes and diminished epidermal adhesion. Most COL17A1 mutations described lead to premature termination codon and cause absence of collagen XVII in the skin. In contrast, missense mutations or deletions of collagen XVII are more rare and the genotype-phenotype correlations of these mutations are poorly understood.

Here we report two novel glycine substitution mutations, G609D and G612R, which are located in the largest collagenous domain, Col15, of collagen XVII. The effects of the mutations on the triple-helical structure and function of collagen XVII were analyzed by generating mutations in an expression vector coding for full-length collagen XVII using site-directed mutagenesis. The mutated collagens were expressed by transfection in COS-7 cells and their thermal stability was assessed using trypsin digestions at increasing temperatures as a probe. Both glycine substitutions G609D and G612R destabilised significantly the ectodomain of collagen XVII which manifested as about 16°C lower Tm (the midpoint of the helix-to-coil transition) in the trypsin assay. This is comparable to effects of two other, previously published mutations, G627V and G633D, which decreased the Tm of the ectodomain by 20°C. Thus glycine substitution mutations of Col15 domain interfere with the proper tight folding of the triple-helix and render the ectodomain of collagen XVII sensitive to non-specific degradation.

093 [Oral 044]

Contact of High-Invasive but not Low-Invasive Melanoma Cells to Collagen I Induces Increased Release of Pro- and Mature Cathepsin B

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The expression of the lysosomal cysteine protease cathepsin B in malignant tumors is highly upregulated and the localization of the protease is altered. In addition to its localization in perinuclear vesicles, previous studies indicate that cathepsin B is also secreted and becomes associated with the cell surface. Tumor cells secrete procathepsin B and both active forms of cathepsin B, but yet the mechanisms underlying these processes are not well understood. Therefore, we investigated to which extent the interaction of tumor cells with the extracellular matrix component, collagen type I, regulates the expression and especially the release of cathepsin B. Four melanoma cell lines (MV3, SkMel28, SkMel23, WM164) differing in their invasiveness were cultured for 48h either as monolayers or in 3D collagen I lattices. After a 24h serum-free incubation period the supernatants were analyzed via immunoblotting. Low-invasive cells (SkMel23 and WM164) showed no remarkable release of cathepsin B, even after they were seeded into collagen I lattices. SkMel28 cells (intermediate-invasive) released both active forms of the protease after contact to collagen I. Cultured as monolayers, only the high-invasive MV3 cells constitutively released procathepsin B. Due to cell-collagen I interaction the amount of released proform was strongly increased and the release of both active forms was induced. Furthermore, we could assess the proteolytic activity of released cathepsin B by the use of our recently described technique of gelatine zymography under acidic conditions. The zymogramms also demonstrated that the melanoma cell lines release in addition other active lysosomal cysteine proteases, but to a lesser amount when compared to cathepsin B. Based on these results we propose, that release of mature cathepsin B is non-selective and a consequence of lysosomal exocytosis induced by the interaction of invasive melanoma cells with collagen I. Increased release of procathepsin B is exclusively observed in high-invasive cells and might be due to alterations in normal trafficking pathways enhancing their invasive capacities in the extracellular matrix.

094

Induction of Lysyl Hydroxylase 2 and Formation of Skeletal Type Collagen Cross-links in the Skin of Systemic Scleroderma Patients: a Potential Role for Interleukin-4?

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Sclerotic skin diseases like lipodermatosclerosis or keloids are characterized by substantial changes in the mode of cross-linking of collagen molecules. This process leads to an accumulation of hydroxylysine-derived cross-links in the skin which are typical for skeletal tissues. In the present study, we analyzed the crosslink pattern and the transcriptional activity of collagen modifying enzymes in systemic scleroderma. Furthermore, we determined the modulatory impact of interleukin 4, which has been implicated in the pathogenesis of systemic scleroderma, on the profile of cross-links.

The concentrations of hydroxylysine-derived collagen cross-links were significantly increased in systemic scleroderma. However, the concentrations of lysine-derived cross-links were not changed. Accordingly, a marked increase of the transcriptional level of lysyl hydroxylase 2 was found, while the gene expression of lysyl oxidase, lysyl hydroxylase 1 were unchanged. In long-term dermal fibroblast cultures, interleukin-4 induced an increase of hydroxylysinederived collagen cross-links and of the gene expression of lysyl hydroxylase 2.

The cross-link pattern of collagen in systemic scleroderma is characterized by an increase in hydroxylysine-derived cross-links, which are typical for skleletal tissues. This change in cross-link pattern appears to be due to an increase in the transcriptional activity for lysyl hydroxylase 2, and can be induced in cell culture by interleukin-4.

095

Expression of Transforming Growth Factors β and their Receptors in Keloid and Hypertrophic Scarring

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Keloids are benign skin tumors occurring during wound healing in genetically predisposed patients. They are characterized by an abnormal deposition of extracellular matrix components, in particular collagen. There is evidence that transforming growth factor-beta (TGFβ) is involved in keloid formation. It is unclear how TGFβ signalling is connected to the pathogenesis of keloids. Therefore we investigated the expression of TGFβ1, 2 and 3 and their receptors in keloids, hypertrophic scars and normal skin.

Dermal fibroblasts were obtained from punch-biopsies of patients with keloids, hypertrophic scars and normal skin of healthy individuals. Total mRNA was isolated and the expression of TGFβ1, 2 and 3 and of TGFβ receptors I and II (TGFβRI and II) was analysed by real-time PCR using the Lightcycler technique. Biopsies of keloids, hypertrophic scars and normal skin were used for immunohistochemistry staining with antibodies against TGFβ1, 2 and 3 and TGFβRI and II.

Our data demonstrate significantly higher TGFβ2 mRNA expression in keloid fibroblasts as compared to fibroblasts derived from hypertrophic scars ($p < 0.05$). By contrast, significantly lower TGFβ3 mRNA expression was found in keloid fibroblasts in comparison to hypertrophic scar derived fibroblasts ($p < 0.01$). TGFβRI mRNA expression was significantly decreased in hypertrophic scar fibroblasts ($p < 0.01$) and TGFβRII mRNA expression was decreased in keloids compared to normal fibroblasts ($p < 0.001$). The ratio of TGFβRI/TGFβRII expression was decreased in keloid fibroblasts. As recently supposed a decreased TGFβRI/ TGFβRII ratio could promote fibrosis.

Therefore our data support a possible role of TGFβRI and TGFβRII as fibrosis inducing factors in keloids.

096

Tretinoin Pre-Treatment but not Direct Treatment Shows a Beneficial Effect on Wound Healing in Diabetic Mice

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There is circumstantial evidence that topical pre-treatment with tretinoin (all-trans retinoic acid) might improve the healing of human ulcers. Especially in diabetic patients with long lasting ulcers the delayed healing process is a persistent problem. We investigated and compared the effect of tretinoin pre-treatment and direct treatment on impaired wounds in genetically diabetic mice. Wounds of db/db mice are display delayed wound healing due to lessened wound contraction and delayed granulation tissue formation.

In first experiments, mice were pre-treated with tretinoin for 5 consecutive days and after a 2 day pause incisional wounds were made on the back of the mice. The tretinoin treated skin of the mice showed severe signs of irritation such as redness and intense scaling. A significant increase in the tensile strength of the tretinoin treated animals compared to the vehicle treated ones was observed 5 days after wounding. Furthermore, positive effects on skin thickness, wound contraction and collagen density were detected upon histological examination.

In further experiments the direct treatment of incisional wounds with tretinoin was examined. Treatment with tretinoin started the same day as wound setting and was continued for 5 consecutive days. Tensile strength was measured on day 7 and day 10 after wounding. In contrast to the results obtained with tretinoin pre-treatment, a decrease of tensile strength in the tretinoin treated animals was observed in the direct treatment of existing wounds.

The differing results of the two treatments are assumed 1) to be due to the irritation caused by the retinoid that counteracts the beneficial effect of tretinoin and 2) to be the result of treatment kinetics: when treatment and wounding start at the same time, positive effects, like thickening of skin, increase of collagen and wound contraction, set in too late to be beneficial in this wound healing model.

In conclusion, the incisional wound healing model with pre-treatment of the skin of db/db mice can be used to investigate the effects on tensile strength of retinoids and their derivatives, of which those lacking the extremely irritating properties of tretinoin would be the most promising drug candidates for ulcers that are often observed in diabetic patients.

097 [Oral 062]

Pathological Scar Cells Fail To Undergo a Form of Apoptosis Specifically Induced During Collagen Gel Contraction – Role of Tissue Transglutaminase Activity

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Keloid and hypertrophic scarring are severe excessive scars that commonly arise during cutaneous wound healing. The exact aetiology is unknown. These pathological scars are characterised by excess collagen and prolonged presence of myofibroblasts. Normally, towards the end of the fibroproliferative phase myofibroblasts disappear via apoptosis. A failure of pathological scar cells to undergo this process may well underlie their pathology. Published studies that examine this possibility give contradictory findings, however, the methodologies used had little relevance to the wound-healing context. The normal trigger for this "switching off" process is unknown, however recently, Fluck *et al* described a potential role for fibrillar collagen in the regulation of apoptosis; where dermal fibroblasts seeded into contractile but not anchored collagen gels underwent apoptosis. We therefore hypothesised that pathological scar cells fail to undergo this specific form of wound-healing-related apoptosis. Fibroblasts derived from normal scar ($n = 8$), hypertrophic scar ($n = 6$) and keloids ($n = 6$) were placed into 3D-collagen (type I) gels and maintained anchored or allowed to contract the gels, and apoptosis was assayed. Although normal scar cells underwent significant ($p < 0.001$) apoptosis (40–50%) after 3 days, all pathological scar cells failed to undergo apoptosis under these circumstances. Furthermore this phenomenon was found to be specific since pathological scar cells could be induced to undergo apoptosis by multiple other modalities. We further hypothesised that this phenomenon might be due to the matrix of pathological scar being refractive to enzymatic breakdown, since chronic fibrotic tissue is excessively crosslinked and hypertrophic scars overexpress tissue transglutaminase (tTgase), an enzyme that stabilises new ECM. We therefore manipulated tTgase activity in cell-containing collagen gels using putrescine to inhibit endogenous enzyme activity or the addition of exogenous tTgase to increase activity, and found that tTgase inhibition in HTS cells normalises their behaviour of collagen contraction-induced apoptosis. Whereas, treatment of collagen gels with exogenous transglutaminase completely abrogates collagen-contraction-induced apoptosis of normal scar cells.

098 [Oral 063]

Passive Transfer of Human Extracellular Matrix Protein 1 Autoantibodies From Lichen Sclerosus Sera Into Neonatal Mice

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The precise immunopathological basis of lichen sclerosus (LS) is not known. However, we have demonstrated presence of IgG autoantibodies to the glycoprotein ECM1 in ~75% of LS sera. Moreover, antibody titre, as determined by ELISA, correlates to some extent with disease severity and duration. Despite these findings, there are currently no data about the possible pathogenic relevance of these anti-ECM1 antibodies. In this study, we performed passive transfer experiments using affinity-purified IgG from LS sera ($n = 6$). Individual mice ears were injected intradermally every 4 days for up to 28 days, either with anti-ECM1 antibodies or with non-immune human IgG as controls. Biopsies were taken at different time points up to 28 days. In the anti-ECM1 antibody-injected sites distinct histological changes were evident from 14 days onwards. These comprised oedema, epidermal acanthosis, dilated dermal blood vessels, and interstitial and perivascular chronic inflammatory infiltrates. These dermatopathological abnormalities were not present in control injected ear skin. IF labelling with FITC-conjugated anti-human IgG showed positive staining in the lower mouse epidermis and around blood vessels, i.e. at sites corresponding to the known distribution of ECM1 in mouse and human skin. Thus, although the passive transfer of human anti-ECM1 IgG antibodies did not fully recapitulate the LS histopathology (e.g. no hyalinosis) within the time-frame studied, we were able to show that this human IgG can access native ECM1 *in vivo*. In short, this study provides the first direct evidence for the possible role of humoral autoimmunity to ECM1 contributing to the disease process/mechanism underlying LS.

099 [Oral 065]

Elucidation of the Antifibrogenic Effector Mechanisms of Alpha-Melanocyte-Stimulating Hormone

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Melanocortins elicit a plethora of biological effects on skin cells including pigmentation, immunomodulation, modulation of oxidative stress, and lipogenesis. We recently reported that human dermal fibroblasts express functional receptors for alpha-melanocyte-stimulating hormone (alpha-MSH). Application of alpha-MSH suppressed the fibrogenic effect of transforming growth factor-beta1 (TGF-beta1) on collagen synthesis *in vitro* and *in vivo* (Böhm *et al*, J. Biol. Chem. 2004). In contrast to artificial cAMP inducers which antagonize the effect of TGF-beta1 in rat fibroblasts by suppressing collagen synthesis at the transcriptional level, alpha-MSH in human dermal fibroblast does not act in an analogous manner. TGF-beta1-induced nuclear translocation of smad2/3 is unaffected by alpha-MSH and alpha-MSH does not affect ligand-induced smad2 phoshorylation in these cells. In addition, time-kinetic studies on smad7 using real-time PCR failed to demonstrate any modulatory effect of alpha-MSH on this anti-smad member. As demonstrated by high-density oligonucleotide arrays using Affymetrix HuGeneFL gene chips and validation by real-time PCR analysis, alpha-MSH, however, modulated the expression of a number of effector/target molecules involved in fibrogenesis including members of the matrix metalloproteinase family (i.e., MMP1, MMP19) growth factors (e.g. basic fibroblast growth factor) or intracellular signalling mediators (e.g. inducible nitric oxide synthase). Taken together, our data show that alpha-MSH does not affect TGF-beta1-induced smad signalling in human dermal fibroblasts being consistent with its previously reported lack of any effect on collagen type I transcription. In contrast, alpha-MSH may elicit its antifibrogenic effects by targeting other effector/target molecules of TGF-beta1, some of which are crucially involved in degradation of collagen.

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(-)-Muscone and Saffron Extract Selectively Regulate Hyaluronan Synthesis at the MRNA Level in Human Skin Cells

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Hyaluronan (HA), a high-molecular-weight linear glycosaminoglycan, is well known for its ability to hold water and maintain the extracellular space. The HA content in the skin is reported to decline with age, possibly contributing to wrinkle formation and the decreased elasticity of aging skin. Among the family of HA synthase genes (*HAS 1, 2, 3*) so far identified, our group has demonstrated that the expressions of *HAS2* and *HAS3* play crucial roles in the regulation of HA synthesis in human skin fibroblasts and keratinocytes, respectively, but the precise regulatory mechanisms are still unknown. In the present study, we discovered that two novel regulators, (-)-muscone and saffron extract, selectively regulate the *HAS* gene expressions in human skin cells.

(-)-Muscone, a major odoriferous constituent of natural musk, up-regulates HA synthesis in cultured human skin fibroblasts specifically and dose-dependently, but it exerts no such effect in cultured human keratinocytes. Saffron extract, the extract of the dry stigmas of the plant *Crocus sativus*, dose-dependently enhanced HA synthesis in both keratinocytes and fibroblasts, though to a smaller degree in the latter. In Northen blot analysis, (-)-muscone and saffron extract induced the expressions of *HAS2* and *HAS3* mRNAs in fibroblasts and keratinocytes immediately after the stimulation, respectively. It thus appears that both (-)-muscone and saffron extract control HA synthesis at a transcriptional level but do so via different mechanisms, the former exerting an effect for *HAS2* expression in fibroblasts, the latter exerting an effect specific for *HAS3* expression in keratinocytes.

In previous studies, we demonstrated that TGF-β1 up-regulates HA synthesis in fibroblasts by inducing the expression of *HAS* mRNAs, generally *HAS2* mRNA[1]. For this reason, we hypothesized that the regulation of *HAS2* expression by (-)-muscone might involve a TGF-β signaling pathway. To elucidate this possibility, we investigated the effect of (-)-muscone on collagen synthesis upregulated by TGF-β1 in fibroblasts. Failing to find any increase in collagen production in response to the (-)-muscone stimulation, we speculated that (-)-muscone might control the HA synthesis via a mechanism unrelated to that of TGF-β1.

On the other hand, the active ingredients of saffron extract are now under investigation.

Further study focusing on the controls of *HAS2* expression by (-)-muscone in fibroblasts and *HAS3* expression by saffron extract in keratinocytes may lead to the discovery of a new system for the regulation of HA synthesis in human skin fibroblasts.

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Isometric Tensile Forces Stimulation of Human Dermal Fibroblasts by Hydroxyprolin Rich Glycoproteins Extract

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Myofibroblasts (MF), a specific phenotype of human dermal fibroblasts have the ability to develop tensile strengths inside the dermal extracellular matrix due to a well-developed cytoplasmic microfilament network particularly rich in α-smooth muscle actin (α-SMA). Dermal Internal tension forces have been previously shown to influence the texture of skin tissues. The present work investigates the effects of an hydroxyproline rich hydrolyzed soy flour extract (SF) treatment on (i) isometric tensile strengths developed by dermal equivalents (DE) and (ii) human fibroblast differentiation into myofibroblasts. DE composed of human dermal fibroblasts and type I collagen were placed in the GlaSbox® registration chamber allowing measurement of isometric forces. On SF treated DE, isometric tensile forces increased in a dose dependent manner from 2000 mN (control) to 3000 mN and 4000 mN with 0.75 mg/ml and 1.5 mg/ml SF respectively. In the same time, TGF-β1 (2.5 ng/ml), a potent inducer of MF phenotype also stimulated the tensile strength to 5000 mN.

In order to confirm the role of MF in the stimulation of isometric tensile forces, DE were immunostained for α-SMA and an increase in α-SMA positive fibroblasts in SF and TGF-β1 treated DE was shown. In addition, SF treated human dermal fibroblasts monolayer cultures revealed a strong dose dependant increase in α-SMA up to 1.75 times at SF 1.5 mg/ml. These results demonstrate that the stimulation of the isometric tensile strengths of dermal equivalent by an hydrolyzed soy flour extract is mediated by myofibroblasts and that this extract may be proposed to reinforce dermal mechanobiological properties and matrix remodelling.

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A First Missense Mutation in the Type II Hair Keratin hHb3 is Associated with Monilethrix

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Monilethrix is an autosomal dominant disorder characterized by periodic beading of hair shafts and pronounced hair fragility leading to hair loss and eventual scarring alopecia. Several familial cases of monilethrix were previously shown to be caused by mutations in the basic (type II) hair keratins hHb1 and hHb6. In the related type II hair keratin family member hHb3, disease-causing mutations have so far not been described. Here we report a first heterozygous missense mutation associated with monilethrix in exon 7 of *hHb3* leading to the substitution of a glutamic acid by a lysine (E407K). The *hHB3* mutation is located in the keratin helix termination motif, which is a mutational hotspot in *hHb1* and *hHb6*. In two other patients with a typical monilethrix phenotype, one was associated with a known mutation in hHb6 (E402K) while no mutations in hHb1, hHb3 or hHb6 were found in the other patient.

Our findings show for the first time that besides hHb1 and hHB6, also hHB3 is associated with monilethrix. In addition, one of our patients indicates genetic heterogeneity in monilethrix, which confirms previous data from other studies.

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A Compound Heterozygous Mutation in VEGFR3 Does Not Cause a More Severe Form of Nonne-Milroy Lymphedema

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Nonne-Milroy lymphedema or congenital hereditary lymphedema I (MIM #153100) is a chronic progressive, disfiguring and often disabling swelling of the extremities caused by dysfunction of the lymphatic system. Mutations in the *FLT4* gene that codes for vascular endothelial growth factor receptor 3 (VEGFR3) cause this autosomal dominant form of hereditary lymphedema. VEGFR3 and its ligands VEGF-C and VEGF-D are important for the formation of the lymphatic system. Almost all mutations found so far have been heterozygous missense mutations affecting the VEGFR3 tyrosine kinase domains. A genotype-phenotype correlation has not been established, as the phenotype is highly variable.

We report a family with two affected siblings, a two-year-old boy and his eleven-year-old sister. Both children are related by their mother, having different fathers. All parents are clinically unaffected. Examination of the daughter showed a bilateral lymphedema of the lower limbs and feet, next to insufficiency of the saphenofemoral crosse and the greater saphenous vein on both sides, accompanied by prominent phlebectasias on the medial side of the legs. The son is less severely affected than his older sister, having a unilateral lymphedema of the left lower limb and foot. A diagnosis of Nonne-Milroy disease was made.

We screened both affected individuals for VEGFR3 mutations. In both siblings we identified a known nucleotide change (2797 G>C) leading to a G933R substitution. The mutation was inherited from the mother. The son inherited an additional nucleotide change from his father (3145 G>A) leading to a D1049N substitution. Each mutation affects one of the two tyrosine kinase domains, the catalytic part of the receptor.

It is striking that the phenotype of the compound heterozygote mutation is less severe than the one caused by the G933R substitution alone. It is known that the G933R mutation has a variable penetrance. One possibility might be that the D1049N mutation interferes with the effect of the G933R substitution. Since primary lymphedema is a progressive disease, difference in phenotype could also be an age-related effect. However, when she was her brother's age, the sister was more severely affected than he is now.

Here, we show that compound heterozygous missense mutations are not associated with a more severe phenotype. Hence, the phenotype of Nonne-Milroy disease may be influenced by genetic background rather than by specific mutations.

To our knowledge, this is the first case of a compound heterozygote mutation in VEGFR3 causing primary congenital lymphedema. Insufficiency of the saphenofemoral crosse and greater saphenous vein due to a VEGFR3 mutation has never been described before. From this and other reports, VEGFR3 seems to be involved in venous patterning as well.

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Hereditary Angioedema in Consequence of a 150 bp Deletion in the 3′UTR of C1-inhibitor Gene

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3′ untranslated regions (3′UTRs) are of great importance in the regulation of gene expression. Situated between the termination codon of a gene and it's poly-A-tail, specific sequences modulate cleavage of the transcript by endonucleases influencing translation, transport and stability of the transcript. Furthermore it contains binding regions for a number of trans-acting proteins possessing translation enhancing functions. Rarely, the significance of these cis-and trans-acting factors has been demonstrated in pathophysiological models.

We report on a family suffering from hereditary angioedema, of which three members (mother, son and daughter) were analyzed for alterations in the C1-inhibitor (C1-INH) gene. Hereditary angioedema (HAE) is a autosomal dominant inherited disease, characterized by angioedema of face and extremities, as well as episodes of abdominal pain and oedemas of the laryneal mucosae. Reduced levels of functional C1-inhibitor protein due to mutations leading to a quantitative reduction of the protein or the synthesis of dysfunctional molecules can be found as the cause. All exons of the C1-INH gene were analyzed by means of polymerase chain reaction (PCR) and subsequent sequence analysis, revealing a heterozygous 155bp deletion located in the 3′UTR, 100bp downstream of the termination codon in exon 8. No other mutations could be detected in the coding region of the gene. Expression analysis at the cDNA-level revealed a clear reduction of C1-INH cDNA. The mutation was not found in 30 control cDNA's. Reduced protein levels of C1-INH protein and C4 was demonstrated in all three patients. Therefore a correlation between the deletion in the 3′UTR and the expression level of the C1-INH mRNA respectively can be assumed, leading to haploinsufficiency of the respective enzyme levels. This deletion constitutes the largest deletion in the 3′UTR of a gene leading to pathological condition.

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Identification and Expression of Two Novel Splice Variants of Human p38α

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The p38α gene plays an important role in regulating the development and proliferation of cells and in response to disease and tissue injury. Four alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported. We hypothesised that this gene should have more splice variants which are differentially expressed in response to environmental stress and proinflammatory cytokines. Here we report the identification and partial characterization of two novel splice variants of p38α, namely p38α-v5 and p38α-v6, by RACE-PCR using mRNA derived from foreskin-derived human primary keratinocytes. Comparative analysis of intron/exon organization of all the six p38α variants revealed that the two novel variants each contains a different exon 1 which is located within the intron 1 of p38α. The distinct transcriptional starting sites (TSSs), resulting from the distinct exon 1 among p38α-v5, p38α-v6 and the other four known isoforms, suggest that the p38α gene has at least three distinct promoter regions in response to different stresses. The full-length cDNA of p38α-v5 is the same as that of MAPK14 transcript variant 2 (or CSBP2) except for its exon 1 and contains an open reading frame of 849 bp encoding a N-terminus-truncated CSBP2 of 283 amino acids. The full-length cDNA of p38α-v6 contains an open reading frame of 1005 bp which is predicted to encode an 335-amino-acid isoform that has a different N-terminus as compared to CSBP2. RT-PCR indicated that they showed different tissue distribution unlike the ubiquitous expression of p38α within the tested 18 human tissue samples. Both of p38α-v5 and p38α-v6 are expressed in the spleen, thymus, small intestine, bone marrow, stomach, lung skin, and bronchial epithelial cells but not in the tonsil, tongue, polyp, salivary gland, or nasal epithelial cells. However, in contrast to p38α-v6, p38α-v5 is also expressed in the neutrophils, colon and uterus whereas but p38α-v6 is in the adenoid and tracheal epithelial cells. Real-time RT-PCR revealed that there are only low expression changes observed in the tissues mentioned above as well as in cultured human primary keratinocytes stimulated with TNFα, IL-1β, H_2O_2, phorbol–myristate-acetate, *P. aeruginosa* culture supernatants, and MAPK inhibitors SP600125, SB202190 or PD98059. These results indicate that relatively parallel expression level among p38α-v5, p38α-v6 and the other four variants are crucial in a specific tissue. This is the first report on the identification of two novel splice variants of p38α and the demonstration that the p38α gene exists with different TSSs. Our findings may help to elucidate in more detail its protein function and imply that it can be regulated through its transcription induction in addition to its protein activation by the MKKK-MKK-MAPK phosphorylation module or TAB1-mediated autophosphorylation.

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Identification of 900 Genes Expressed by the Human Granular Keratinocyte

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Investigation of the late transcriptionnal events taking place in the course of epidermis terminal differentiation is critical to comprehension of cornification and barrier function. Transcriptome analysis of granular keratinocytes is a first step towards such goals. We isolated four cell populations, from basal to the most superficial epidermal layers, by repeated trypsin incubations of human epidermis fragments. The analysis of KRT14 (specifically expressed in the basal layer) and SCCE (specifically expressed in the granular layer) mRNAs by quantitative RT-PCR in each cell batch confirmed the progressive enrichment in granular keratinocytes.

Starting with cell batch corresponding to the most superficial layers we obtained 2000 high-quality Open Reading Frame EST (ORESTE) and clustered them with public sequences to produce an index of approximately 900 genes that includes 100 genes encoding hypothetical proteins. Analysis of gene expression levels by EST frequency identifies genes that characterize squamous differentiation thus providing potential markers as well as clues to the functional features of cornification.

A complete analysis of expression of this set of genes in the course of epidermis differentiation will allow us to distinguish the genes expressed in all epidermis layers that might correspond to house keeping genes, from the late expressed genes that are likely to encode proteins candidate for being involved in barrier function. The EST recovered in this work will provide a comprehensive resource for genes functioning in establishment of the epidermis barrier function.

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Functional Characterization of PRINS, a Newly Identified Psoriasis Susceptibility Related, Stress-Induced Non-Coding RNA Gene

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In order to identify transcriptional level differences between psoriatic non-involved and healthy epidermis contributing to psoriasis susceptibility, differential display experiments were carried out. One of the identified transcripts that showed elevated expression in psoriatic non-involved epidermis had 100% homology to a cDNA sequenced from a human 10-week-old foetal cDNA library, with GeneBank Acc. No. AK022045. This gene is localised on chromosome 10, harbours two Sq subtype *Alu* elements and *in silico* translation resulted in no translatable protein product on its nucleotide sequence. Using either the pol II inhibitor, α-amanitin or the specific pol III inhibitor, tagetitoxin, we have demonstrated that PRINS is an RNA polymerase II transcribed gene. Because this gene harbours Sq subtype *Alu* elements, that is characteristic of RNA genes induced by stress, we named the full-length cDNA PRINS that stands for Psoriasis-susceptibility Related RNA Gene Induced by Stress. To study whether PRINS expression was affected by different stress conditions, *in vitro* cultures of HaCaT immortalised keratinocytes were subjected to various stresses and the expression level changes of PRINS were measured by Q-RT-PCR. PRINS expression was induced by the translational inhibitor cycloheximide, and by HSV-1 infection, also known to cause translational inhibition. Upregulation by translational inhibitors is characteristic of genes containing *Alu* elements (such as PRINS) and may be part of a general stress response. Co-incubation with microbial compounds, such as lipopolysaccharide, tuberculin, peptidoglycane and *Candida albicans* resulted in elevated PRINS expression that peaked after 12 hours incubation with each compound. UV-B irradiation of HaCaT keratinocytes increased the expression of PRINS that also peaked 12 hours after the irradiation. Serum factors also strictly regulate the expression of PRINS. RNA silencing experiments in HeLa cells revealed that silencing of the PRINS gene expression affects the survival of the cells, indicating its protective role in stress response.

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Differential Processing of Dominant Mutants Of Connexin 26 In Transgenic Mouse Epidermis Is Associated With Distinct Phenotypes

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An increasing body of evidence indicates that intercellular communication via gap junctions plays an important role in regulation of epidermal differentiation and growth. Dominantly acting mutations in genes encoding gap junction proteins, connexins (Cx) 26, 30, 31 and 30.3, are associated with skin disease but underlying pathomechanisms remain unclear. While Cx26(D66H) causes a severe palmoplantar keratoderma (Vohwinkel syndrome), Cx26 (del 42E) causes a milder form. To investigate the mechanisms of action, the two Cx26 mutants were expressed in cultured keratinocytes and in the suprabasal epidermis of transgenic mice, employing a K10 promoter. While Cx26(D66H) failed to traffic to the plasma membrane and accumulated in the Golgi apparatus of cultured epithelial cells, Cx26(del 42E) trafficked normally and formed large junction plaques at intercellular contacts, although these gap junctions did not transport the tracer Lucifer yellow-CH. Similarly, when expressed in the mouse epidermis, Cx26(D66H) accumulated in keratinocyte cytoplasm, while Cx26 (del 42E) accumulated in plasma membranes, although overall abundance of the mutated connexins appeared similar, as assessed by immunofluorescence staining. These differences in intracellular distribution were associated with subtle differences in phenotype of the transgenic mice. Commencing at postnatal day 4, K10-Cx26(D66H) mice showed a severe keratoderma of trunk, tail and limbs, progressing on the tail to constriction rings which caused autoamputation. In contrast, K10-Cx26(del 42E) mice showed no phenotype until postnatal day 7, when a series of distinct kinks appeared in the tail; these persisted until about postnatal day 14. The kinks were associated with bands of epidermal hyperkeratosis restricted to the dorsal side of the tail. Thus it appears that Cx26(D66H), when expressed in suprabasal keratinocytes causes a severe, widespread hyperkeratosis, while Cx26(del 42E), which assembles into gap junction plaques, has a more subtle effect, manifest in transgenic mice only by the disturbance of epidermal differentiation during the period of rapid tail elongation. These new transgenic mice will provide useful models for detailed analysis of the mechanisms underlying the phenotypic effects of different Cx26 mutants and whether these mechanisms involve direct disturbance of the epidermal gap junction network or of other properties of endogenous connexins. Particularly intriguing is the question of whether the tail autoamputation caused by Cx26 (D66H) or the kinking caused by Cx26 (del 42E) are the direct results of disrupted epidermal structure or are secondary to altered release of epidermal factors which influence the growth of underlying connective tissue and bone.

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Transcriptional Responses to Conventional and Pegylated Interferon-alpha Inhibiting Tumor Growth in a Human Melanoma SCID Mouse Xenotransplantation Model

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Interferon-alpha (IFN-α) is widely used for the treatment of viral infections and primary cancers. In the present study we investigated whether the anti-proliferative activity of IFN-α is capable of inhibiting melanoma tumor development in the absence of protective immune responses in a SCID mouse model. Mice treated with either regular (100 μg/ 3 times per week) or pegylated (300 μg/ once weekly) human IFN-α 2a showed a marked reduction in tumor weight after 4 weeks of treatment. Tumor weight in pegylated and conventional IFN-α treated animals was reduced by 61% and 67% respectively as compared to saline control (both $p \leqslant 0.01$). A decrease of proliferation and an increase of apoptotic tumor cells were observed in IFN treated tumors.

DNA microarrays were applied to analyse transcriptional responses in tumors after 4 weeks of treatment and a subset of about 90 genes was differentially expressed. 24 novel and 5 known interferon inducible genes were up- and 65 genes down-regulated.

A direct comparison of IFN-α and pegylated IFN-α did not reveal any significant differences in tumor growth inhibition indicating that this novel and more stable class of IFN's is functionally equivalent. Despite the structural difference between pegylated and conventional IFN-α both agents caused similar transcriptional responses in human melanoma xenotransplants.

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UPAR Inhibition in Melanoma Cells by Small Interfering RNAs

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The plasminogen activator system affects invasion and metastasis of several cancer types. In melanoma, the expression of the urokinase-type plasminogen activator (uPA) and its receptor (uPAR) is increased in advanced stages and positively correlates with the invasive potential of melanoma cells. Moreover, interference with uPA/uPAR expression or function reduces invasion and/or metastasis in animal models of melanoma. For further characterization of the role of the plasminogen activator system in melanoma, we have developed small interfering RNAs (siRNAs) for uPAR as a molecular tool and as a potential anti-metastatic strategy. Suitable siRNA target sequences were identified in the uPAR mRNA, and siRNAs were designed. Effective transfection into the metastatic melanoma cell line 1205Lu was established with an FITC-labeled siRNA and flow cytometry. Basal uPAR surface expression as assessed by flow cytometry was reduced 24 h after transfection of uPAR siRNAs, whereas sequence-controlled siRNAs had no effect. Additionally, PMA induction of uPAR was prevented by siRNA resulting in a 75% reduction of uPAR expression compared to PMA-induced cells treated with the control siRNA. The inhibitory effect was confirmed at the mRNA level by quantitative RT-PCR showing up to 90% reduction of basal and 70% reduction of PMA-induced uPAR mRNA expression. Taken together, we designed uPAR siRNAs that effectively and specifically inhibit uPAR expression in a melanoma cell line. The siRNAs will be utilized to further investigate the role of uPAR in melanoma invasion and metastasis and might lead to the development of a new molecular therapy for melanoma.

111 [Oral 014]

The 3′ Untranslated Regions of the Two Alternative Type XVII Collagen mRNA Transcripts Have Different Effects on Translation Efficiency

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Type XVII collagen is a transmembrane component of the hemidesmosome coded for by the *COL17A1* gene. Transcription generates two alternative mRNA's both with the full coding sequence, but with different 3′ untranslated regions (3′UTR's). The difference is a deletion of 610 nucleotides, which results in a transcript of 5.8 kb and a smaller transcript of 5.2 kb. The ratio of the two transcripts varies between different cells. While in normal human keratinocytes and skin the larger 3′UTR is dominantly present, it is the minor transcript in the squamous cell carcinoma line UMSCC-22B. Compelling evidence suggests that 3′ UTR sequences might have various regulatory functions involving among other things translational control and cell development. We have investigated whether the two different type XVII collagen mRNA 3′UTR's have different effects on the translation efficiency of the preceding coding sequence. Plasmids were constructed with the 3′UTR's cloned behind the firefly luciferase coding sequence. These constructs were transfected in several cell lines and firefly luciferase protein expression was measured by adding luciferase substrate, which results in a stable luminescent signal. Renilla luciferase plasmids functioned as internal expression markers. Luciferase expression appeared very low in cells transfected with the smaller 3′UTR in comparison with cells transfected with luciferase plasmids with the entire 3′UTR. This different effect on expression was the same irrespective if type XVII collagen positive or type XVII collagen negative keratinocyte cell lines were transfected. These results suggest that the type XVII collagen 3′UTR's are involved in translational control and that keratinocytes are able to direct the type XVII collagen protein level.

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Genotype-Phenotype Correlation for Ectodermal Dysplasia Syndromes and the Tail of *p63*

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More than 170 different forms of ectodermal dysplasia (ED) have been described, many of which remain a molecular mystery. However, some autosomal dominant ED syndromes have been shown recently to result from mutations in the gene encoding the p63 transcription factor. p63 is expressed in basal keratinocytes and in embryonic apical ectodermal ridge and is believed to have a dual role in the development and maintenance of the epidermis. The tail region of p63, encoded by exons 13 and 14 of the gene, contains a sterile alpha motif (SAM), relevant to protein-protein interactions, and an inhibitory domain. Moreover, a small number of mutations in this region have been shown to underlie ankyloblepharon, ectodermal dysplasia, clefting (AEC) syndrome and Rapp-Hodgkin (RH) syndrome. In this study, we assessed genotype-phenotype correlation by screening for *p63* mutations in 12 subjects with a clinical diagnosis of AEC syndrome and two with RH syndrome, all from unrelated families. All AEC individuals had heterozygous missense mutations in the SAM domain. These comprised: G505T, L514F, L518V, C522W, C522G, G530V, T533P, Q536L (twice), I537T (twice) and L545P. In the two RH patients, one had a heterozygous missense mutation, S541P, while the other had a heterozygous frameshift mutation, 1787delG, in exon 14. This frameshift leads to a delayed termination codon, changing the reading frame of the last 46 amino acids and extending the p63 tail by a further 68 amino acids. Clinically, AEC and RH syndromes share many clinical features including cleft lip/palate, hypohidrosis, and a characteristic facies with a narrow nose and a small mouth. An erosive scalp dermatitis and ankyloblepharon may favour a diagnosis of AEC syndrome, but there appears to be a spectrum of clinical signs common to both disorders. Moreover, this study shows that the clinical syndromes are associated with overlapping *p63* gene pathology. Most cases of AEC syndrome appear to have missense mutations in the p63 tail and this also occurs in RH syndrome, although the delayed termination codon may be a feature of RH syndrome. Clearly, genotype-phenotype correlation will need to be extended in further patients, but these initial data suggest that AEC and RH syndromes may well be the same clinical entity, sharing similar molecular pathologies.

113 [Oral 046]

Allele Variations in the *OCA2* Gene (Pink-Eyed-Dilution Locus) are Associated with Genetic Susceptibility to Melanoma

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The *OCA2* gene, localized at 15q11, encodes a melanosomal transmembrane protein that is involved in the most common form of human occulo-cutaneous albinism, a human genetic disorder characterized by fair pigmentation and susceptibility to skin cancer. We wondered whether allele variations at this locus could influence both human pigmentation characteristics and susceptibility to melanoma (MM). Ten intragenic single nucleotide polymorphisms (SNPs) were genotyped in 113 patients with melanomas and in 105 Caucasian control subjects with no personal or family history of skin cancer. The effect of *OCA2* on susceptibility to MM and on pigmentation characteristics was assessed using both a classic statistical method (comparison of SNPs allelic frequencies) and the recently developed "combination test". We show by both statistical approaches that MM and *OCA2* are associated (respective p values = 0.0056 and 0.030 after correction for multiple testing), suggesting that the risk of melanoma is determined by several SNPs, possibly interacting, on *OCA2*. In addition, the *OCA2* genotype was also found to be strongly associated with eye color ($p = 0.0035$ after correction for multiple testing, OR = 5.58 [1.96–16.7]). Finally, the effect of *OCA2* on melanoma risk persisted after stratification for eyes color, and was also detected using a logistic model taking into account the *MC1R* genotype, a gene strongly involved in MM and all pigmentation characteristics. Our data demonstrate that a second pigmentation gene, in addition to *MC1R*, is involved in susceptibility to melanoma and in human pigmentation.

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Mutations of the RNA-specific Adenosine Deaminase Gene (*ADAR1*) are Involved in Dyschromatosis Symmetrica Hereditaria

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Dyschromatosis symmetrica hereditaria (DSH, reticulate acropigmantation of Dohi) has been reported mainly in Japan, although it occurs in families of every ethnic origin all over the world. The patients have pinpoint, pea-sized hyper- and hypo-pigmented macules on the back of the hands and the top of the feet. These abnormalities are otherwise asymptomatic and do not affect the general health. DSH generally shows an autosomal dominant pattern of inheritance with high penetrance. Many clinical and morphological investigations have been reported, but the cause and the pathogenesis have not yet been clarified. We succeeded in mapping and positionally cloning the *DSH* gene. We performed a genome-wide search in three families (41 affected and 47 unaffected), and mapped the *DSH* locus to chromosome 1q21.3. To further refine the localization, we identified novel single nucleotide polymorphisms and integrated the genetic and physical maps of the region. The final genetic interval was approximately 500kb in which 9 genes ware mapped. To detect the pathological mutations, affected individuals from each of the 3 pedigrees named Pedigree 1, 2 and 3, plus new pedigree named Pedigree 4, were screened by PCR-SSCP and direct sequencing. The results showed that they were heterozygous for mutation alleles of double-strand RNA-specific adenosine deaminase gene (*ADAR1*), R474X, L923P, K952X and F1165S respectively. None of these four mutations were found in 55 unaffected individuals in any of the four pedigrees or in 116 unrelated normally pigmented Japanese adults that were surveyed. Thus, we concluded that those four mutations are not polymorphic but are the pathologic ones causing the disease. Additionally, we identified seven novel mutations, H216ins.CC, K433ins.TG, R426X, Q600X, P727del.C, V906F and K1201del.A in new seven cases of DSH. We compared the clinical features among the patients with the mutations identified in eleven cases, but no clear correlation was established between genotypes and phenotypes. And the eleven kind of mutations identified in our studies did not suggest any founder effect. ADAR catalyses the deamination of adenosine to inosine in double-stranded RNA substrates that result in the creation of alternative splicing sites. We speculate that the failure of correct RNA editing may induce differentiation of melanoblasts to hyper- or hypo-active melanocytes colonizing in an irregular distribution in the skin lesions during the development.

115 [Oral 067]

Altered Expression of Actin and Focal Adhesion-Associated Proteins in Kindler Syndrome

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Kindler syndrome (KS; OMIM173650) is an unusual, autosomal recessive skin disorder associated with trauma-induced blisters in early life followed by photosensitivity, poikiloderma, and an increased risk of malignancy. Recently, defects in the actin/focal adhesion associated protein kindlin (also known as kinderlin) encoded by the gene *KIND1* have been shown to cause this disease. In human epidermis, kindlin is expressed in epidermal keratinocytes, particularly within basal keratinocytes and at the dermal-epidermal junction (DEJ). We have undertaken a detailed ultrastructural and immunohistochemical study in KS ($n = 2$) and control skin ($n = 3$) to examine DEJ morphology and the labeling patterns of various basement membrane, actin cytoskeletal and focal contact-associated proteins. Transmission electron microscopy of KS skin showed disruption and reduplication of the lamina densa, together with sub-lamina densa cleft formation. The number and structure of hemidesmosomes and anchoring filaments appeared normal, although there was focal disruption in desmosome- and hemidesmosome-keratin filament attachment. This disruption in normal keratin filament assembly was most obvious at sites of dermal clefts and was associated with an abundance of substratum-associated, disorganized bundles of actin filaments. Immunofluorescence microscopy showed increased epidermal expression of actin, α actinin, talin, vinculin, tenascin C and RACK-1 in KS skin but no change in labeling with antibodies to filamin, tensin, focal adhesion kinase, paxillin or tropomyosin. Immunostaining for protein kinase C was markedly reduced in basal keratinocytes in KS skin compared to control. Taken together, our findings reveal a close spatial and functional relationship between kindlin, actin, some focal contact proteins and regulatory molecules that link the actin skeleton to the integrin extracellular matrix receptors. We hypothesize that the function of kindlin might be to bind to the terminal ends of actin microfilaments, linking focal adhesion proteins to the integrin receptors, thereby limiting the elongation of actin microfilaments. Conversely, a lack of kindlin might disrupt focal adhesion linkage and allow the unregulated proliferation of actin microfilaments causing perturbations in the associated actin and keratin cytoskeletal networks.

116 [Oral 002]

***Spink5* knockout Mice Demonstrate a Key Role for Lekti in Epidermal Integrity**

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SPINK5 is the defective gene in the severe autosomal recessive ichthyosiform skin condition, Netherton syndrome. *SPINK5* encodes the putative multi-domain serine protease inhibitor LEKTI, which is a marker of epithelial differentiation, strongly expressed in the granular layer of the epidermis. To investigate its role *in vivo*, we have genetically engineered mice with a targeted disruption of *Spink5*. The targeted locus was confirmed by Southern blot hybridization. Loss of expression of Lekti was demonstrated by absence of immunostaining in the epidermis of the knockout mice. Homozygous mutant mice displayed a marked skin fragility with superficial peeling and died shortly after birth. These lesions were predominant on the limbs, the tail and the trunk. Hair development was impaired, resulting in the absence of erupted vibrissae and hair canal. The visible scarce hair shafts were misoriented and had an altered curving. Histological analysis of the vibrissal follicle demonstrated loss of intercellular contacts in the inner root sheath and a shrunk hair shaft. Ultrastructural examination of the epidermis showed detachment between the granular layer and the stratum corneum. Intercellular spaces in the upper granular layer presented with abnormal globular material and split desmosomes. Stratum corneum detachment led to a severe skin barrier defect as measured by penetration of external dyes and increase of transepidermal water loss. Our study provides the first *in vivo* evidence for a role of *Spink5* in the desquamation of the epidermis through the regulation of cell-cell adhesion between terminally differentiated keratinocytes. These results also demonstrate that *Spink5* is essential for hair development. *Spink5* knockout mice features are very close to Netherton syndrome and therefore provide a useful model to elucidate the pathophysiological pathways of Netherton syndrome.

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Development of Potential Microbicides Based on RNA Interference of DC-SIGN

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In order to develop a microbicide based on RNA interference, downregulation of cellular receptors for HIV attachment and transfer must be achieved. DC-SIGN mediates HIV attachment on dendritic cells that are the first targets during the course of HIV infection as well as transfer to CD4+ T cells. For this purpose, we developed lentiviral vectors that express short hairpin RNA (shRNA) for the delivery of small interfering RNA (siRNA) capable of conditionally suppressing DC-SIGN expression. Selectivity of inhibition on DC-SIGN, L-SIGN, chimpanzee or Rhesus macaque DC-SIGN was obtained by the use of distinct siRNAs. Suppression of DC-SIGN expression inhibited the attachment of the gp120 envelope glycoprotein of HIV-1 to DC-SIGN transfectants, as well as transfer of HIV-1 to target cells *in trans*. Furthermore, shRNA-expressing lentiviral vectors were capable of efficiently suppressing DC-SIGN expression in primary human DCs. DC-SIGN-negative DCs were unable to enhance transfer of HIV-1 infectivity to T cells *in trans,* demonstrating an essential role for this receptor in transferring infectious viral particles from DCs to T cells. The present system is a first step towards the use of RNA interference of DC-SIGN as potential microbicide for HIV as well as other pathogens also recognized by this receptor such as HCV.

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Gene Expression Profiling of Skin and Lymph Nodes of Rats Affected with Contact Hypersensitivity

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The transcriptional changes in skin and draining lymph nodes of rats during the elicitation phase of contact hypersensitivity (CH) were examined. Female Sprague Dawley rats, sensitized against 2,4-dinitrofluorobenzene (DNFB), were challenged with 0.5% DNFB on the flanks and the involved skin and the draining lymph nodes dissected 8 or 24 hrs after the challenge. The challenge resulted in an increase by 1.1 mm (8 hrs) and 0.9 mm (24 hrs) in skin thickness and by 2.5 mg (8 hrs) and 26.8 mg (24 hrs) in lymph node weight. The RNA from the affected tissues and from corresponding tissue samples from naïve animals (controls) was extracted and hybridized on GeneChip microarrays (Affymetrix RU34A, 8799 gene probe sets). Genes which were significantly differently expressed with $p \leq 0.001$ in challenged mice and in controls were analyzed. From 1882 genes retrieved 309 genes exhibited a 3-fold different expression pattern at 8 or 24 hrs after challenge. Clustering these 309 genes revealed 117 genes up-regulated in skin (S+) and 114 genes down-regulated (S−) whereas in lymph nodes 19 genes were up- (L+) and 31 genes were down-regulated (L−). Twenty eight genes were up-regulated in both tissues (SL+). Among those genes, genes related to signal transduction [e.g. Tle-4 (L+), Ptpn-16 (L+), Jak-2 (S+)], apoptosis [e.g. Casp-3 (S+), Casp-2 (SL+), Dnase31 (S−)], stress [e.g. Hsp-70 (S+), Mapk-9 (SL+)], extracellular matrix [e.g. Mmp-3, -19, -12, -13(S+), decorin (L−), Col1α1 (L+)], cell adhesion [C-CAM-4 (S+), Tnc (S+), Sell (S+)], and genes related to inflammatory reactions [as CD14 (SL+), MRP-8 (SL+), iNos (S+), MRP-14 (S+)] as well as genes of interleukins and chemokines [as IL-6 (S+), IL-1α (S−), Gro-1 (S+), MCP-1 (S+), IP-10 (SL+)] were detected. Their expression levels was in most cases time-dependent. The validated transcriptional signatures of the responses of involved tissues will give insights into the diverse molecular cascades in CH which underlay the cellular events and result in allergic contact dermatitis.

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Genetic Correction of Xeroderma Pigmentosum : New Approaches Compatible with Patients Therapy

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Xeroderma pigmentosum (XP) is a rare hereditary disease transmitted with autosomal and recessive traits. XP patients are characterized by an extreme photo-sensitivity and a high predisposition to skin cancer development from keratinocyte origin. Nucleotide excision repair (NER), the most versatile repair mechanism of UV-induced DNA lesions, is deficient in XP cells. Seven classical complementation groups of XP cells have been identified and corresponding genes (*XPA to XPG)* have been cloned.

In the absence of any efficient pharmacological treatment, our objective is to restore *ex vivo* the repair capacity of XP keratinocytes after genetic correction using retroviral vectors. As a first attempt, small surface epithelial sheets obtained from genetically corrected XP keratinocytes will be engrafted onto consentent XP patients. To this end, a first set of encouraging results have been obtained in the laboratory, demonstrating for the first time full phenotypic reversion of the XP-C phenotype using classical retroviruses expressing the *XPC* DNA repair gene and the G418 resistance gene (*neo*). Corrected keratinocytes recovered normal DNA repair and cell survival capacities as well as proliferation and differentiation defects previously revealed upon the first reconstuction of XP-C skin *in vitro*.

To refine this approach and in order to fulfil therapeutical requirements and compatibility with XP patients, we have now developed a new generation of retroviral vectors. In these vectors, the *neo* gene has been replaced by a natural cell surface marker of post-mitotic/suprabasal epidermal keratinocytes, CD24. After transduction using a LTR-CD24-ires-GFP retrovirus, CD24 could be ectopically expressed at the surface of proliferative/basal keratinocytes. Using an anti CD24 antibody and magnetic beads enabled us to selectively select in a single step 98% of transduced keratinocytes and also fibroblasts. 100% of cells selected for CD24 expression also stably expressed the GFP marker. On this basis, vectors encoding *XPC* instead of GFP were constructed and are currently used to revert the phenotype of XP-C keratinocytes and then to reconstruct *in vitro* genetically corrected XP skin. Our strategy should help minimizing immune responses connected to the expression of exogenous resistance markers, thus opening the first realistic prospect of retrovirus-mediated cutaneous gene therapy of the XP.

120 [Oral 068]

***In vitro* Reconstruction of Skin From Nevoid Basal Cell Carcinoma Syndrome Reveals Impact of *PATCHED* Mutations in Cutaneous Homeostasis**

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Gorlin syndrome, or naevoid basal cell carcinoma syndrome (NBCCS), is a rare autosomal dominant disorder characterised by a range of developmental abnormalities and a high proneness to the major type of non melanoma skin cancer, basal cell carcinoma. In most cases, clinical halmarks of Gorlin syndrome results from mutation of the *PATCHED* gene which encodes the putative transmembrane receptor of SONIC HEDGEHOG (SHH). In the general population, the role of PATCHED in the maintenance of epidermal homeostasis has remained poorly documented. Also, it is still unclear how germinal mutation of *PATCHED* may result in the high predispostion to BCC of NBCCS patients. Ultraviolet and gamma irradiations have often been evoked as possible etiological agents of BCC development in NBCCS patients. Recently, we provided evidence that ultraviolet irradiation represses *PATCHED* gene transcription, suggesting that genotoxic stresses impact the SHH/PTC pathway. In the present study, we generated primary fibroblasts and keratinocytes strains from skin of consentent NBCCS patients harboring a known germinal *PATCHED* mutation. We determined cell survival and DNA repair capacities of NBCCS epidermal keratinocytes toward UVB and gamma irradiations and showed that these cells are neither photosensitive nor radiosensitive. Using NBCCS fibroblasts and keratinocytes, we successfully reconstructed NBCCS skin *in vitro*. At first sight, the stepwise process of stratification appeared normal. However, presence of NBCCS fibroblasts in the dermal equivalent and of normal keratinocytes in the epidermal compartment led to the formation of frequent clefts at the dermal epidermal junction. The occurrence of clefts was exaggerated in the presence of NBCCS cells in both cutaneous compartments. Labelling of molecules involved in cohesiveness between basal keratinocytes and basement membrane revealled a stricking, spontaneous increase of laminin and beta-1 integrin. Numerous apoptotic keratinocytes could be revealed above these mechanically fragilized regions. These results suggest the crucial role of abnormal mesenchymal/epithelial interactions in exacerbated predisposition of NBCCS patients toward BCC development. Currents investigations aim at the determinations of molecular clues involved in this newly documented phenotype of the NBCCS skin reconstructed *in vitro* in the absence or following UVB irradiation.

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Expression Profiles of Inflammatory Skin Diseases: results of a SAGE™ and Microarray Analysis

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As part of a large-scale study, we have generated the disease specific expression profiles of skin affected with psoriasis, atopic and contact dermatitis, or Lichen planus, and healthy skin by SAGE™ analysis. In addition, we performed an initial microarray analysis using PIQOR™ Skin microarrays. The SAGE™ analysis revealed altogether 201 differentially expressed genes when compared to healthy skin ($p < 0.01$). Among these genes, 25 were specific for psoriasis, 23 for atopic dermatitis, 24 for contact dermatitis, and 52 for Lichen planus. The suitability of those genes as universal marker genes or drug targets will be validated by microarray experiments of single patient samples, as the SAGE™ libraries were generated based on patient pools. An initial set of 10 microarray experiments per indication proved that clustering of diseased and healthy skin on basis of whole skin biopsies is possible. The stable and reproducible results confirmed the suitability of our protocols for biopsy excision, storage and analysis. When we compared the results of the two expression profiling methods for 120 genes that showed a significantly altered expression in at least one indication in the SAGE™ analysis, we found a good overall concordance of SAGE™ and microarray data. The exhaustive analysis of skin disease specific expression profiles, correlated with medical histories and histopathology data will allow the stratification of patients and help to develop new diagnostic and prognostic tools for skin diseases.

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Possible New Complementation Group(s) of Xeroderma Pigmentosum

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Xeroderma pigmentosum (XP) is an autosomal recessive photosensitive disease with abnormal pigmentation and an extremely high incidence of skin cancers. Cells from patients with XP are hypersensitive to killing by ultraviolet (UV), which is associated with impaired ability to repair UV-induced DNA damages. There are genetically different seven nucleotide excision repair (NER) deficient groups (A ~ G) and a NER proficient form (XP variant). We have been receiving more than 200 clinical samples for the diagnosis of XP for these five years and 70 patients were newly diagnosed as having XP in our laboratory. Among them, we found three unusual Japanese XP cases (XP29HM, XP30HM and XP36HM). Those patients (17 y.o. female, 35 y.o. male and 55 y.o. male) are not related and have mild clinical features with a few skin cancers in areas exposed to sunlight and so far any evidence for neurological abnormalities was not detected. Fibroblasts derived from those patients were hypersensitive to UV irradiation compared to cells from normal subjects and less sensitive to UV than XP-A XP-C and XP-D cells, while the levels of post-UV unscheduled DNA synthesis (UDS) in those patients were 26%, 23% and 12% of normal, respectively. Complementation test by cell fusion technique and a plasmid host cell reactivation assay revealed that those patients did not belong to XP group A, B, C, D, F and G. DNA repair studies and gene analysis indicated that those patients were not in XP group E and XP variant. Cell fusion analysis using those three cell strains implied that there were at least two more new XP complementation groups. Molecular and biochemical analyses using the cells from those patients should give a new insight in NER pathway.

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Defining the Transcriptional Control Elements that Regulate Keratinocyte-Specific Expression of *p63*

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The development and differentiation of the mammalian skin epidermis is governed by a number of critical transcription factors including p63, a homolog of the tumor suppressor p53. Recent gain-of-function and loss-of-function studies in mice have underscored the important role of p63 in sustaining the progenitor cell populations in stratified epithelium of the skin epidermis and in maintaining the balance between proliferation and differentiation. Interestingly, by utilizing alternate promoters, the *p63* gene gives rise to 2 classes of transcripts: transcripts, which encode for an amino terminal transactivation domain (TA) and transcripts that lack this domain (N). The expression of these two isoforms is spatio-temporally regulated during development of the embryonic mouse skin. In addition, it has been postulated that keratinocytes make choices to proliferate, differentiate or stratify based on the relative levels of these two isoforms. However, very little is known about the transcriptional control mechanisms that govern the expression of p63 in keratinocytes. In order to identify the *cis*-elements that regulate p63, we have analyzed the cell-type specific chromatin conformation of the mouse *p63* gene by DNase I hypersensitive site mapping. These studies along with cross-species genome sequence comparisons have allowed us to uncover critical regulatory domains that are important for directing the expression of p63 in keratinocytes. We have performed functional assays of these regulatory domains by testing them for transcriptional activation in transient transfection experiments and in transgenic mice. These studies have identified a critical regulatory segment that harbors most of the transcriptional activity of the p63 promoter. Molecular and biochemical characterization of these elements by Electrophoretic Mobility Shift Assays and mutation analysis has revealed critical roles for members of several well-studied transcription factor families. Collectively, our data provide novel insights into the complex regulatory mechanisms that control the expression of p63 isoforms during epidermal development and differentiation.

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Laminin-5 Mutation Survey and Genotype-Phenotype Correlation in Italian Patients with Junctional Epidermolysis Bullosa

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Junctional epidermolysis bullosa (JEB) is a rare genodermatosis characterised by dermal-epidermal separation and caused by mutations in the genes encoding hemidesmosomal components and laminin-5, the major epithelial adhesion ligand. We have examined a cohort of 19 Italian JEB patients presenting defective laminin-5 expression, 11 of them affected with the lethal Herlitz (H JEB) and eight with the mild non-Herlitz variant of JEB (non-H JEB). JEB patients were diagnosed on the basis of clinical features, immunofluorescence antigen mapping and ultrastructural examination of skin biopsies. Mutational analysis of the *LAMA3A*, *LAMB3* and *LAMC2* genes encoding the three chains of laminin-5 was performed by heteroduplex analysis of genomic PCR fragments or by RT-PCR and direct sequencing of the amplified cDNA products. Through these screening methods, an overall mutation detection sensitivity of 95% was obtained. Eighteen mutations, seven of which novel, were identified and their consequences analysed at the mRNA and protein level. H JEB patients carried premature termination codon (PTC) mutations in 20 out of 22 mutant alleles, with a prevalence of mutations in *LAMC2*, whereas splicing or missense mutations compatible with a reduced synthesis of mutant laminin-5 were prevalent in non-H JEB patients. However, in one case, a homozygous PTC mutation in *LAMB3* was associated to illegitimate splicing leading to non-H JEB. In addition, we characterised a large intragenic duplication within *LAMC2* in a H JEB patient, a genetic defect so far uncovered in laminin-5 genes. Collectively, five mutations appeared to be frequent in laminin-5 JEB patients (R635X, 29insC, E210K, W143X in *LAMB3* and R95X in *LAMC2*), two of which (R95X and W143X) being so far detected only in patients from southern Italian regions. These recurrent mutations account for approximately 44% of laminin-5 JEB alleles in Italian patients: screening at first for these mutations will allow to accelerate molecular diagnosis in new Italian JEB patients.

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Expression Profiling of Melanoma Correlates Active TGFβ Signaling with Molecular Patterns of Increased Metastatic Potential

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Melanoma is an aggressive tumor originating from melanocytes either in normal skin or within melanocytic nevi. Despite growing awareness of the disease the incidence of melanoma continues to increase world-wide. Most importantly, melanoma is recognized as being the most dangerous of skin cancers. Once metastasis has been confirmed patient prognosis is extremely poor, and successful treatment has hitherto relied solely on removal of the primary tumour before the onset of invasive metastasis.

We have recently conducted a series of large-scale DNA microarray experiments on a subset of established human melanoma cultures. The raw data (44500 probe-set signal intensity measurements per sample) was normalized, filtered using fold-change criteria and then used for hierarchical clustering. Clustering revealed that the molecular signatures of the melanomas were distributed among three well defined cohorts, these cohorts were termed *quiet*, *intermediate* and *active* according to their increasing molecular signature divergence from the *control* (melanocyte culture) group. Analysis of the available literature strongly suggests that the *quiet* and *active* cohorts are representative of melanomas which are, respectively, poorly and strongly metastatic. Additionally, clinical data indicates an improved survival for patients yielding *quiet* cohort melanomas.

The original normalized dataset was refiltered using a statistical (ANOVA) selection for probes which distinguish the three cohorts to reveal 2819 cohort-specific probe-sets. The signal intensity expression patterns of these, across the full range of melanomas and melanocyte controls, was used for self-organizing map (SOM) clustering in order to distinguish the main expression pattern groups. Extensive literature analysis of the principal genes composing one of three major expression pattern motifs, for which genes are upregulated in the *intermediate* and *active* cohort melanomas, show that TGFβ signaling is being activated in these melanomas. Incubation of *quiet* cohort melanomas with exogenous TGFβ shows that this signalling pathway is intact. This suggests that there is a TGFβ signal block mechanism which is active in *quiet* melanomas and not (or less so) in the *intermediate* and *active* cohorts.

Our current work focuses on the hypothesis that increased metastasis is driven in part by a reduction in TGFβ signal control.

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Novel COL7A1 Mutations in Dystrophic Epidermolysis Bullosa and Genotype-Phenotype Correlations

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Dystrophic epidermolysis bullosa (DEB), a hereditary skin disorder characterized by subepidermal blistering, is caused by mutations in the type VII collagen gene, COL7A1. Molecular diagnostics of DEB remain complex due to the large size of COL7A1, large variety of mutations and heterogeneity of the phenotypes. Here we report the mutations of 20 central European patients with various DEB forms and genotype-phenotype correlations. All 118 COL7A1 exons were amplified from genomic DNA by PCR and submitted to automated sequencing. The expression of mutated collagen VII was examined by RT-PCR, IF-staining and immunoblotting, allowing the assessment of genotype-phenotype correlations. We found 5 novel glycine substitutions: G1522R (4564G>C), G1525R (4573G>A), G1616R (4846G>A), G2689R (8065G>A), G2737R (8209G>A) situated in exons 45–51 and 109–110. All lead to reduced levels of collagen VII in the skin, as shown with IF staining. The mutations were associated with different clinical phenotypes. Furthermore, a novel splice site mutation 426+1G>A was disclosed in intron 3 donor position +1. As recurrent mutations, the non-population-specific, frequent splice site mutation 425A>G and the nonsense mutation R1933X (5797C>T) were identified. These data allow us to set up an efficient strategy for COL7A1mutation analysis in central European patients. Further studies on protein level will contribute to better understanding of the molecular pathomechanisms leading to the different clinical and biological phenotypes.

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Expression of PRINS, a Newly Identified Non-Coding RNA Gene in Psoriasis and in Various Human Tissues

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Using differential display we have recently isolated a transcript that is overexpressed in psoriatic uninvolved epidermis. The gene coding for this transcript is localised on chromosome 10 and *in silico* translation resulted in no translatable protein product on its nucleotide sequence. Sequence analysis revealed that the gene harbours two Sq type *Alu* elements and *in vitro* experiments with immortalised HaCaT keratinocytes demonstrated that the expression of this gene is upregulated under different stress conditions. Therefore, we named the full-length transcript Psoriasis-susceptibility Related RNA Gene Induced by Stress, PRINS. In order to check the tissue specificity of PRINS, its expression was tested by Q-RT-PCR in samples from 16 different human organs and tissues. Our results showed that PRINS was expressed in all human tissues examined and the abundance of PRINS mRNA showed big differences in the various tissues (lowest in cardiac muscle, highest in veins). Reverse-Southern and Q-RT-PCR experiments proved that the expression of PRINS was 25 times higher in the non-involved epidermis of psoriatic patients (n=5) compared to healthy epidermis (n=10). However, the expression in psoriatic plaques (n=8) was only 8 times higher than in healthy epidermis. When normal (n=5) and psoriatic non-involved (n=5) epidermises were treated with a T-lymphokine mixture, that has been showed to induce a hyperproliferative response in non-lesional, but not in normal keratinocytes, the expression of PRINS in normal epidermis was not affected, at the same time PRINS expression in psoriatic non-involved epidermis was downregulated to the level of expression found in the psoriatic lesional epidermis. In synchronized HaCaT keratinocytes, PRINS was substantially down-regulated in proliferating, and upregulated in differentiated HaCaT keratinocytes. The overexpression of PRINS in the psoriatic non-lesional keratinocytes may reflect an altered regulatory extracellular milieu, but it is also possible that PRINS plays a regulatory role in the hyperproliferation in psoriasis.

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Stress Response in Epidermis from Patients with Keratin Genodermatoses invokes Phosphorylation of Hsp27 at Serine 82

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Epidermal strength and resilience is dependent on expression and organisation of keratin intermediate filaments (IF), a major component of the cellular cytoskeleton. However, keratin mutations produce altered proteins that weaken structural stability in a dominant negative manner and so cause a variety of genodermatoses affecting the epidermis and its appendages. Stress placed on epithelial cells causes collapse of the IF network which leads to blistering, hyperproliferation, and hyperkeratosis. Stress initiates a cellular protective response and various molecular chaperones are up-regulated and activated. In human epidermis, a small heat shock protein family member (Hsp27) has a specific affinity for keratin IF proteins and activation of Hsp27 by phosphorylation at serine 82 is observed in normal granular cells. We have now demonstrated increased phosphorylation of Hsp27 at serine 82 as part of the stress response to IF collapse and cellular disruption in several genodermatoses. Eight cases of clinically and genetically characterized genodermatoses (2 EBS, 2 EHK, 1 EPPK, 2 IBS and 1 LEN) and 3 normal volunteers were studied. Serial 5 μm sections of paraffin embedded tissue were treated with mouse mAbs to Hsp27 or Hsp27-p82 (detects phosphorylated active form) and visualised with DAB. In all eight cases, an increase in Hsp27 expression was observed compared to normal as well as a dramatic increase in levels of phosphorylated Hsp27 (p82). This activated form is normally limited to the granular layer but general and intense staining was observed in all suprabasal cells. We conclude that the cellular disruption caused by keratin IF collapse invokes a stress response that leads to the up-regulation and specific phosphorylation of Hsp27.

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Association of Apolipoprotein E Polymorphisms with Psoriasis

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Apolipoprotein E (apoE) is a lipid transport protein which regulates the metabolism of cholesterol and endogenous and dietary triglycerides (TG). Hyperlipidaemia, particularly hypertriglyceridaemia, changes in lipoprotein composition and a high prevalence of cardiovascular disease are well documented in patients with psoriasis. ApoE was elevated in 31 Chinese psoriasis patients and one specific polymorphic apoE variant showed association with psoriasis in a Japanese cohort of patients. Three common alleles, ε2, ε3 and ε4, defined by exonic single nucleotide polymorphisms (SNPs) T3937C and C4075T, encode the three major isoforms E2, E3 and E4. ApoE2 is protective against hyperlipidaemia and cardiovascular disease, whereas the apoE4 isoform is associated with high cholesterol and is over represented in hyperlipidaemic and heart disease populations.

Our aim was to identify any novel SNPs in Caucasian psoriasis patients and investigate the frequency of coding SNPs T3937C and C4075T in patients with chronic plaque psoriasis (CPP) (n = 212), guttate psoriasis (GP) (n = 94), palmoplantar pustular psoriasis (PPP) (n = 101) and healthy controls (n = 137). DNA was obtained from patients and controls and genotyped using a radioactive hybridisation technique. Allelic frequencies were analysed by the chi-squared test.

Only SNPs defining ε2, ε3 and ε4 were detected by DNA sequencing of 30 patients with severe CPP. Patients with CPP and GP showed significantly increased frequency of the + 3937C SNP (19.6% and 17.80%, p = 0.002 and 0.02) and the ε4 allele (3937C/4075C) (18.40% and 18.10%, p = 0.008 and 0.029) compared with controls (10.8% and 10.9% respectively). There was no significant difference between the + 4075C and + 4075T SNPs in patients and controls. CPP and GP show strong association with PSORS1 (in contrast to PPP). When CPP and GP cases were combined the + 3937 C SNP (p = 0.002) and the ε4 allele (p = 0.006) were significantly more frequent in cases (19% and 18.3%) than controls [OR 1.82 (1.18–2.80)]. There was no significant difference in apoE SNP or allele frequencies between PPP patients (10.5% and 10.7%) and controls.

These data demonstrate that the apo ε4 allele is more common in individuals with psoriasis and suggest a possible pathogenic role of the apoE lipoprotein. Furthermore, the increased frequency of the apo ε4 allele in psoriasis may account for the high prevalence of cardiovascular disease observed in these patients.

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Therapeutic Potential of Placenta Growth Factor in Diabetic Wound Healing

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Placenta growth factor (PlGF) is a member of the vascular endothelial growth factor (VEGF) family that has been found to play an important role in adult angiogenesis. Such feature has led to consider PlGF as a potential candidate for therapeutic modulation of angiogenesis, and PlGF treatment of ischemic tissues proved beneficial in inducing angiogenesis and collateral vessel growth by mobilizing/recruiting bone marrow-derived precursors cells. In cutaneous wound healing PlGF is expressed by migrating keratinocytes and proliferating endothelium during the angiogenic phase, and wound closure is delayed in PlGF-deficient mice indicating that this factor plays a role in skin repair. In this study, we have tested the therapeutic potential of PlGF in streptozotocin-induced diabetic mice, as a model of altered skin repair associated with reduced angiogenesis. We first addressed the involvement of PlGF in diabetic cutaneous wound healing by analyzing PlGF expression at different time points after injury. We found that PlGF induction was greatly reduced in diabetic mice compared to non diabetic controls, both at the mRNA and protein level. We then investigated the potential role of PlGF in improving wound healing in transgenic mice overexpressing this factor in the skin under the control of the human keratin 14 promoter (K14-PlGF transgenic mice), rendered or not diabetic by streptozotocin treatment. While K14-PlGF mice did not show an improved healing compared to wild-type controls, in K14-PlGF diabetic mice wound healing was significantly accelerated compared to diabetic non transgenic littermates. We finally investigated the effect of treatment with an adenoviral vector carrying the human PlGF-1 gene (AdCMV.PlGF) topically applied on excisional wounds in diabetic mice. AdCMV.PlGF significantly improved the healing process and accelerated wound closure compared to the control vector (AdCMV.LacZ) or saline. The histological analysis of wound specimens, taken 7 days after injury, revealed that granulation tissue formation was delayed in AdCMV.lacZ-treated and saline-treated diabetic mice compared to Ad.CMVPlGF-treated mice. Further, immunohistochemical PECAM /CD31 staining showed that the average area of the vessels within the granulation tissue was significantly increased in AdCMV.PlGF-treated mice compared to controls. All together our findings underline the importance of the angiogenic factor PlGF in cutaneous wound healing and indicate that PlGF gene transfer may have a therapeutic potential for the treatment of diabetic ulcers.

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Examination of Single Nucleotide Polymorphisms of the Fibroblast Growth Factor Receptor 2 Gene in Patients with Leg Ulcer

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Fibroblast growth factors (FGFs) have potent mitogenic activity to the vast majority of epithelial cells through specific binding to fibroblast growth factor receptors (FGFRs). Thus FGFs and their receptors are important mediators of a wide variety of biologic activities including wound healing. The aim of our study was to identify single nucleotide polymorphisms (SNPs), which may play a role in the pathomechanism of prolonged wound healing in patients with leg ulcer. There have been 397 SNPs identified on the full length FGFR2 genomic sequence from which we chose 5 for further studies that may have effect(s) on gene function. For the SNP analysis experiments we have used the Assay-on-Demand kit of Applied Biosystems. Comparing the SNP data of 73 leg ulcer patients with 71 healthy individuals we could not find significant difference in the distribution of alleles between the two groups (Chi^2 test $p > 0.05$) concerning the SNP in the promoter region (2753T → C), in exon 5 (103A → G, Thr → Met), in exon 6 (64C → T, Val → Val) and the SNP in intron 6 (91C → T). However, the SNP located in the 3′ untranslated region (UTR) of the mRNA (2451A → G; 900 bp downstream from the ORF) showed significant difference in the allelic distribution between patients suffering from leg ulcer and healthy individuals (Chi^2 test $p = 0.01$). We hypothetize that this SNP might alter the stability of the mRNA, results in decreased FGFR2 protein amount, thus to receptor disfunction that leads to prolonged wound healing in patients with leg ulcer.

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Polymorphisms in the Promoter of the Interleukin-4 Receptor Alpha Chain Gene are Associated with Atopic Dermatitis and Psoriasis in Japan

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The trait of enhanced IgE responsiveness has been linked to chromosome segment 16p12, where the interleukin-4 receptor alpha chain (IL4R) gene is located. Recently, Ober *et al* suggested that variations outside the coding region of the IL4R gene might influence susceptibility to atopy and asthma. We sought to find promoter polymorphisms of the IL4R gene that might influence the transcription level of the gene. We found six polymorphisms; −3112C > T, −1803 T > C, −327C > A, −326A > C, −186G > A and −184A > G, numbered from the transcriptional initiation site. A case-control association study demonstrated significant association at −3112C > T ($p = 0.0075$), −1803T > C ($p = 0.0034$), −327C > A ($p = 0.00033$), −326A > C ($p = 0.0031$) and −186G > A ($p = 0.0031$) between patients with atopic dermatitis and normal control subjects. Furthermore, we found significant association between psoriasis and IL4R −326A > C and −186G > A variants. For −326A > C the genotype AA is more frequent ($p < 0.0001$), and for −186G > A, the genotype GG is more frequent ($p < 0.0001$) in psoriasis. Interestingly, these allelic associations are the reverse of those we found in Japanese patients with atopic dermatitis. Nevertheless, none of these IL4R promoter variants had any effect on transcriptional activity when tested fused to a luciferase reporter gene. Thus, although we observed strong association between IL4R promoter polymorphisms and atopic dermatitis, the biological mechanism of action of these variants is not yet clear.

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Maternal Germline Mosaicism, *LAMB3* hotspot mutation R635X and Prenatal Testing in Herlitz Junctional Epidermolysis Bullosa

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Herlitz junctional epidermolysis bullosa (H-JEB, OMIM 226700) is a lethal, autosomal recessive disorder characterized by blister formation at the level of the lamina lucida within the cutaneous basement membrane zone. H-JEB is frequently associated with premature-termination-codon (PTC) mutations in both alleles of one of the three genes *LAMA3, LAMC,* or *LAMB3*) encoding the subunit polypeptides α3, β3, γ2 of laminin 5. The majority of the laminin 5 mutations reside in *LAMB3* gene on chromosome 1 (GeneBank accession number L25541). *LAMB3* has a hotspot PTC mutation 1903C > T, R635X in exon 14. The newborn male from healthy, non-consanguineous parents had extensive blistering and demonstrated negative immunofluorescence staining for laminin 5 β3 chain, and relevated tissue separation within lamina lucida of the dermal-epidermal junction, diagnostic for H-JEB. Mutation analysis was performed by amplification of genomic DNA with polymerase chain reaction using *LAMB3*-specific primers, heteroduplex analysis, and direct nucleotide sequencing. For family screening and mutation verification restriction endonuclease digestion (BglII) was applied. We detected a heterozygous hotspot PTC mutation 1903C > T, R635X. The mother of the proband was found to be a heterozygous carrier for this mutation, whereas the mutation in the father remained unknown. Nonpaternity was excluded by use of microsatellite markers from different chromosomes. Based on these results, DNA-based prenatal diagnosis was performed by chorionic villus sampling for subsequent second pregnancy in the family. The fetus was found to be negative of the R635X mutation, indicating that they was phenotypically unaffected. Hapoltype analysis with intragenic *LAMB3* polymorphisms and using microsatellite markers surrounding the *LAMB3* gene showed that the first patient and the fetus carry the same maternal and paternal chromosome 1/*LAMB3* haplotype, without R635X in the fetus. In that case, an unaffected child was predicted and the mother gave birth to a helthy male. We concluded that the mutation is most likely present in a percentage of the maternal germline, responsible for this unusual mode of inheritance in H-JEB.

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Gene Expression Studies in Sezary Syndrome Using Real-Time Polymerase Chain Reaction

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Detailed allelotyping studies have identified two mutually exclusive regions of deletion on chromosome 10q in Sezary syndrome (SS) and mycosis fungoides at 10q23.33–10q24.1 and 10q24.33–10q25.3.[1] Genes mapped to these regions of deletion were identified from the genome database and include the transcription factor *HHEX,* a lymphoid specific helicase (*HELLS*), a tumour suppressor gene (*MXI1*), a member of the apoptotic cascade (*CASP7*) as well as two recently identified genes: programmed cell death 4 (*PDCD4*) and cutaneous T-cell lymphoma tumour antigen (*FLJ10188*). Real-time PCR was performed to assess expression of each gene in SS. Total RNA was isolated from fresh peripheral blood mononuclear cells from 18 SS patients and 5 normal controls. Real-time PCR on cDNA was performed for each of the above genes using assays-on-demand from Applied Biosystems, Foster City, CA, USA. Results were analysed for statistically significant differences in gene expression between each SS sample and the mean gene expression of the 5 normal controls. A statistically significant reduction in gene expression (at the 99% confidence level) was seen in 78% of SS patients with HHEX, 33% with HELLS, 50% with MXI1, 44% with PDCD4, 67% with CASP7 and 61% with FLJ10188 which was independent of disease burden (measured by Sezary count, CD4:CD8 ratio and total CD4 count) and treatment regime at the time of sampling. When the mean gene expression of the SS patients was compared with the mean gene expression of the normal controls using unpaired t-tests, the only gene to demonstrate a significant difference in expression was PDCD4 ($p < 0.05$). Loss of heterozygosity (LOH) analysis using microsatellite markers adjacent to the genes of interest was undertaken in 12 patients in whom gene expression studies were performed. LOH was found in conjunction with under-expression of HHEX in 3 patients, HELLS in one, MXI1 in 4, PDCD4 in 2, CASP7 in 4 and FLJ10188 in 4 patients respectively. All six genes analysed demonstrated aberrant gene expression in SS which in some patients with LOH may be explained by allelic loss alone producing haplotype insufficiency. Additional mechanisms including mutations or epigenetic phenomena (such as promoter hypermethylation) occurring in patients with or without LOH are also likely to be implicated in aberrant gene expression by producing biallelic events.

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Mutation Analysis of Keratin 5 and Keratin 14 genes in Patients with Epidermolysis Bullosa Simplex

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Epidermolysis bullosa simplex (EBS) is a group of blistering skin disorders caused by defects in one of the two keratin genes, *KRT5* and *KRT14*. The disease is typically inherited by an autosomal dominant way. All cases of EBS are characterized by mechanical stress-induced blistering of the skin, as a result of keratin filament abnormality and cytolysis in the basal keratinocytes. Our purpose was to identify mutations underlying the clinical symptoms and to compare with skin histology in order to define a phenotype-genotype correlation more precisely. We have investigated genomic sequences of *KRT5* and *KRT14* for mutations in 11 pedigrees from the register of the Hungarian Epidermolysis Bullosa Center. In five EBS pedigrees the disease inherited autosomal dominantly, however in the other six families only the probands but no other family members were affected. Four patients with the most severe form of EBS, Dowling-Meara, have generalized herpetiform skin blistering beginning within a few days after birth. The other seven patients had Weber-Cockayne subtype of EBS, the milder form with blistering confined to the hands and feet. We report two novel *KRT5* amino acid substitutions (S528G, G543S) both located in the tail domain of the keratin 5, and four nucleotide changes without any amino acid substitution (742C→T, 1602T→C, 1704G→A, 5584T→C). These substiutions add to those previously and provide further evidence of phenotype-genotype correlation in EBS.

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Two Novel Mutations in the *EBP* gene in Conradi-Hünermann-Happle Syndrome

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The X-linked dominant Conradi-Hünermann-Happle (CDPX2, MIM 302960) syndrome belongs to the rare, heterogenous group of diseases called chondrodysplasia punctata. The disease has been recently connected with deficiency of the 3β- hydroxysteroid-Δ8-Δ7-isomerase (EBP), catalysing an intermediate step in the conversion of lanosterol to cholesterol.

The two female proband' s parents are healthy. At birth both children had erythematous, scaly skin symptoms on the back and extremities lasting only for a few months. Patient 1 has had repeated small bone-fractures with epiphyseal stippling at the age of 3, as well as vertebral malformation with secondary scoliosis and shortening of the right leg. We saw the 12-year-old patient because of rare, coarse hair. The diffuse scarring alopecia, the facial dysmorphy, the linear depigmented ichthyosis along the lines of Blaschko and the linear follicular atrophoderma on the arms brought us to the diagnosis of CDPX2. Ultrastructural study of the skin revealed membrane coated empty vesicles in the basal and suprabasal keratinocytes in close connection to keratin filaments, as well as within the keratohyalin granules, further supporting the diagnosis. Patient 2 has been diagnosed to have an asymmetric shortening of the limb in utero, and based on her epiphyseal stippling and mild skin symptoms after birth the diagnosis of CDPX2 was proposed. At the age of 1.5 years mild whorled ichthyosis, follicular atrophic areas, mild patchy alopecia and facial dysmorphy were present.

The entire coding system of the EBP gene was amplified and exon 4 was selected in both patients by heteroduplex analysis for direct nucleotide sequencing. The 321 bp PCR fragment contained in Patient 1 a new heterozygous G-to-A transition at nucleotide position 387, leading to a nonsense mutation at codon 129, confirmed by BsmFI restriction analysis, too. Patient 2 proved to have a G nucleotide insertion at position 406 of exon 4 of the EBP gene, leading to premature termination upstream of the mutation.

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Single Cell Suspensions of Allogenic Keratinocytes as Gene Transfer Vehicle in Wound Healing

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Objectives. Large wounds often require treatment with allogenic skin grafts, if autologous are not available. This will result in rejection of the grafted tissue, leaving epithelial defect.

We investigated whether a single cell suspension of allogenic keratinocytes (KC) applied to porcine full-thickness wounds can serve as a temporary wound coverage and facilitate the formation of a normal epithelium by ingrowths of autologous KC, and serve as gene transfer vehicles.

Methods. 1.5 × 1.5 cm full-thickness wounds were created in five Yorkshire pigs, 4-months old and filled with single cell solutions with either 2.5×10^5 allogenic, autologous, or EGF-transfected allogenic KC. Control wounds received normal saline. Wounds were covered with vinyl chambers, ensuring a wet wound environment. Wound fluid was collected daily, wound contraction was measured every third day. H&E sections from biopsies taken on day 9, 14, 21 and 30 were examined to determine re-epithelialization. EGF in wound fluids was measured by ELISA.

Results. On day 9, re-epithelialization was 50.9% in wounds receiving allogenic KC, 61.8% with autologous KC, 63.3% with hEGF-transfected KC, and 37.3% in saline control wounds. On day 14, 21 and 30 all wounds were 100% re-epithelialized, no defects were present in allogenic KC group. Differences between KC treated groups and the saline control, and between allogenic and EGF-transfected KC wounds were statistically significant. No statistically significant difference could be detected between allogenic or autologous KC nor between autologous or hEGF-transfected KC wounds.

Wound contraction did not differ between the groups.

Wound fluid showed elevated hEGF levels in hEGF-transfected allogenic KC wounds, ranging from 920 pg/ml on day 1 to 37 pg/ml on days 5 to 9. No hEGF expression could be detected in the other wounds.

Conclusion. We were able to demonstrate that a single cell solution of allogenic KC enhanced wound healing in this model. Furthermore, allogenic KC can function as gene transfer vehicles for EGF cDNA and are able to further improve the rate of reepithelialization.

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Lack of Association of Major CARD15 Variants with Psoriasis and Psoriatic Arthritis

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Psoriasis has a strong genetic component in the development of the disease as indicated by familial occurrence and a high concordance rate among monozygotic twins. In genome-wide scan for psoriasis several susceptibility loci have been detected, but the disease causing genes have not yet been identified.

A recent scan, performed on psoriatic arthritis (PsA) which occurs in about 15 percent of psoriasis patients showed a significant locus on chromosome 16 in a region that was already described by genome scan for psoriasis. CARD15, a major susceptibility gene for Cohn's disease (CD) on chromosome 16q, is an interesting candidate gene for psoriasis, because there is a documented clinical association of Crohn's disease with psoriasis, and recently the association of CARD15 mutations with PsA was reported in a Newfoundland population.

We investigated the association of this variant with PsA and the overall psoriasis genotype in our cohort of 360 independent patients with plaque type psoriasis in comparison to 361 age and sex-matched controls. In addition, a second cohort of 89 independent North American PsA patients was included. The diagnosis of psoriasis was made by a dermatologist based on standard clinical criteria. In these patients, PsA was defined as an inflammatory joint disease, negative rheumatoid factor, and lack of another causative condition for arthritis.

Using case-control analysis, the G908R mutation was weakly associated with psoriasis and PsA, but due to the low frequency of this mutation statistical significance was not reached. All other variants inluding leu1007fsinsC and R702W did not show any association with psoriasis or PsA.

In conclusion, a disease causing role for CARD15 mutations could not be confirmed in German or American subjects with PsA.

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Report of a Chinese family with Marie-Unna Hereditary Hypotrichosis and Exclusion of Linkage at 8p21

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Marie-Unna hereditary hypotrichosis (MUHH) is a rare autosomal dominant disorder with progressive hair loss starting in early childhood and aggravating at puberty. Several studies have mapped MUHH gene to chromosome 8p21. Here we reported a Chinese MUHH family with variable phenotypes. All affected individuals have anomalies affecting both hair density and hair shafts. Major Clinical characteristics, disease history and histological examination supported the diagnosis of MUHH. But the features of scarring in this kindred are modest and all patients have no vertex hair loss, which are distinct from typical MUHH. In this study, we performed genotyping and linkage analysis using 11 polymorphic microsatellite markers spanning the MUHH locus at 8p. Two-point linkage analysis using these markers revealed significant exclusion of this locus (LOD scores > -2) at $\theta = 0$. These indicated that there is a range of clinical presentation in MUHH, and more than one genetic locus are responsible for MUHH.

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Antigen-Transduced Dendritic Cells are Superior to Peptide-Pulsed Dendritic cells for the Induction of Cellular Anti-Melanoma Immunity in Mice

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Currently, melanoma vaccines utilizing cultured human dendritic cells (DC) as nature's adjuvant are being tested for patients with metastatic melanoma in early clinical trials. The most effective antigen-loading strategy of cultured DC is still a matter of debate. In the present study we directly compared the efficacy of DC-based melanoma vaccines using synthetic peptides and recombinant adenovirus in an experimental mouse model. We show that adenovirus-transduced are superior to peptide-pulsed DC in mediating protective immunity against experimentally induced metastatic-like growth of B16 melanoma cells in the lungs. We provide evidence that adenovirus-transduced DC present antigen for a much longer time period *in vitro* and stimulated stronger cytotoxic T cell activity *in vivo* than peptide-pulsed DC. We conclude that melanoma vaccines consisting of antigen-transduced DC are significantly more effective than peptide-pulsed DC in an experimental mouse melanoma model. Our results provide a possible explanation for the lack of efficacy in human clinical studies using peptide-pulsed DC and suggest that antigen-loading strategies for DC-based melanoma vaccines need to be optimised.

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Plasminogen Activator Inhibitor 2 (PAI-2) Expression in Netherton Syndrome and Lamellar Ichthyosis: Transglutaminase-1 Up-regulated Versus Transglutaminase-1 Deficient Epidermis

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Plasminogen activator inhibitor 2 (PAI-2), a member of the ov-serpin subfamily, shows a strong presence in the stratum granulosum of the epidermis and in the cornified cell envelope (CE). It is considered to confer protection against apoptosis implicating a substantial role for terminal differentiation. Netherton syndrome (NTS) is a severe congenital ichthyosis with epidermal deficiency of the serine protease inhibitor LEKTI due to mutations in *SPINK5.* Lamellar ichthyosis (LI) is a heterogeneous cornification disorder often caused by autosomal recessive mutations in the transglutaminase-1 gene (*TGM1)*. In at least 50% of all LI patients immunohistochemical studies reveal a thin or absent CE with drastically reduced transglutaminase-1 activity.

We analyzed the epidermal transglutaminase-1 activity and the presence of PAI-2 antigen (#3750, American Diagnostica, Greenwich, CT) in different types of LI compared with NTS, psoriasis vulgaris (PV) and atopic dermatitis (AD). AD skin sections (n = 6) showed normal transglutaminase-1 activity and normal expression of PAI-2 antigen in the stratum granulosum. In contrast, skin from PV (n = 8) and NTS (n = 6) patients displayed a grossly enlarged activity zone of transglutaminase-1 as well as a broadened zone of pericellular PAI-2 expression. Interestingly, epidermis of all 6 patients suffering from transglutaminase-1 deficient LI showed a clear lack of PAI-2 expression or a very faint intracellular immunostaining.

PAI-2 contains three acceptor sites, Gln83, Gln84, and Gln86, for transglutaminase-catalyzed amine incorporation as has been shown for tissue transglutaminase (TGM2) and factor XIIIa. Considering the fact that PAI-2 does not show pericellular staining in transglutaminase-1 deficient epidermis, we propose that PAI-2 is *in vivo* incorporated in the CE by transglutaminase-1 suggesting a potential role in LI and other cornification disorders.

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Stimulation of the Uncoupling Protein 2 Gene, A Fat-Burning Adipocyte Protein

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Eukaryote cells generate ATP in their mitochondria by oxidative phosphorylation. The oxidative reactions of the respiratory chain generate an electrochemical proton gradient across the inner membrane of the mitochondria which is then used by the enzyme ATP synthase to phosphorylate ADP to give ATP. Cell respiration is only partially coupled to ADP phosphorylation in mitochondria. Uncoupling proteins (UCP) are carrier proteins in mitochondria that cause protons to re-enter the matrix, thus destroying the electrochemical proton gradient. The energy liberated is then dissipated as heat and less ATP is synthesized.

UCP2 is abundant in white adipose tissue and can be regulated by fatty acids. UCP2 is thus a potential target for drugs used to treat obesity, as it may promote energy production and diminish metabolic efficiency. Therefore, we have developed a real-time quantitative RT-PCR assay to measure the UCP2 mRNA in 3T3-F442A adipocytes. The quantity of UCP2 mRNA was referred to that encoding the ribosomal S26 protein. The RT-PCR experimental conditions were optimized to detect and quantify 10 copies of the UCP2 transcripts. This assay was checked by measuring the effects of reagents known to influence the transcription of the UCP2 gene. We showed that TNF alpha inhibited quantity, while insulin stimulated UCP2 mRNA transcript number in adipocytes. We used these methods to study the effect of linoleic acid and a polar lipid complex rich in linoleic acid (50%). The amount of UCP2 mRNA was increased by linoleic acid (+49% at 30 μg/ml and + 57% at 60 μg/ml). The polar lipid complex also enhanced the amount of UCP2 mRNA in adipocytes (+214% at 120 μg/ml and +252% at 240 μg/ml).

With a specific, rapid, sensitive and quantitative RT-PCR methods, we identified different molecules which could be modulated the UCP2 transcripts, a fat burning gene, in adipocytes.

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Real Time QRT-PCR Approach for Gene Expression Study on Skin and Different Cutaneous Models

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Gene expression study is one of strategies developed to understand biological processes. Real time quantitative RT-PCR (QRT-PCR) is a suitable tool for studies on differential gene expression and is characterized by wide dynamic range of quantification, high sensitivity and high precision. This technique provides the high accuracy and reproducible results necessary for mRNA quantification experiments.

To investigate cutaneous mechanisms, we have established different QRT-PCR assays on skin biopsies and models like culture cells isolated epidermis, reconstructed epidermis, equivalent dermis models like collagen lattice. All QRT-PCR were performed in conditions to obtain a detection level of 10 copies and an efficacy up to 90% with standard curves and samples.

We studied expression of different genes on the major human skin culture cells: melanocytes, keratinocytes, fibroblasts, adipocytes, dendritic cells. On keratinocytes, we measured the induction of interleukin transcripts (interleukin-1 alpha and 1 beta) with retinoic acid treatment and identified potential retinoic acid like compounds. We studied, on human normal melanocytes, expression of the melanogenesis gene tyrosinase and the inhibitory effect of lactic acid (−43% at 5mM during 24 hours). To study differentiation of epidermis, we compared the expression level of gene on reconstructed epidermis models (RHE) and normal human keratinocyte (NHK) culture. In this way, we showed that involucrin transcripts amount was dramatically increased in RHE than NHK (+1500%).

To study human skin *in vivo*, we developed a RNA extraction protocol and different QRT-PCR from frozen skin biopsies or isolated epidermis. We used this technique to investigate epidermal markers of the photo-ageing process for the face skin. We identified a decrease in expression of interleukin-1beta and beta 1 integrin in photo-aged skin showing immune and structural alterations. Then beta1A integrin transcripts measurement was used to evaluate, *in vivo*, the effect of a cosmetic formula. And we showed that topical application of this anti-wrinkle cosmetic product was able to significantly increase the expression of beta 1 integrin.

In dermis, because of less number of cells and the poor quality of RNA after extraction, expression study *in vivo* was difficult. Fibroblasts cultivated in three-dimensional tissue-like collagen matrix were a good model for investigation. We developed, in this model, a method to detect gene expressed in dermis like talin. We showed that induction of contraction with PDGF was correlated with a decrease of talin amount (−50% at 5 ng/ml during 12 days).

In conclusion, real time QRT-PCR gives way to investigate healthy skin and its alterations and to identify new active compounds.

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Fluconazole Upregulates SconC Expression and Inhibits Sulphur Metabolism in *Microsporum canis*

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Recently we reported that fluconazole downregulates the expression of the fungal metallothionein gene, and increase copper cytotoxicity in *Microsporum canis*, suggesting that besides interfering with ergosterol synthesis, azole derivatives also act on other biological circuits of fungal cells.

We used Differential display to detect gene regulation in fluconazole treated and untreated M. Canis. The fragment is extracted and used as a probe to detect and sequence the gene from cDNA library.

We reports here the isolation and the structural organization of *M.canis* sulphur metabolism negative regulator gene (sconC) an SKP1 family homolog and its regulation by fluconazole. The genomic DNA sequence of 1284 bp *M.canis* sconC contains five introns. Four of them (52, 60, 47 and 70 bp) interrupting the ORF of 724 bp, encoding a protein of 165 amino acids. The fifth intron (56 bp) localized upstream of the ATG transcription initiation sites. Furthermore we found that fluconazole upregulates sconC mRNA expression and protein transcription, and inhibits sulphur metabolism in *M.canis*, most likely due to sconC upregulation. This data confirm our previous conclusion that in addition to its action on ergosterol synthesis, fluconazole acts on other biological pathways in fungal cells. These works help to optimize the antifungal action of azoles.

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Identification of Novel Mutations In Two Hungarian Patients With Hailey-Hailey Disease

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Hailey-Hailey disease (HHD) is a rare hereditary condition characterized by development of blisters at the sites of friction and in the intertriginous areas. Mutations in the ATP2C1 gene have been identified in the background of the disease. The objective of this study was to detect ATP2C1 mutations in Hungarian patients with HHD. PCR amplification of the entire coding region of ATP2C1 was performed. Mutation detection strategies included heteroduplex scanning by conformation-sensitive gel electrophoresis and direct nucleotide sequencing. We found two distinct, heterozygous mutations, both of which were novel. In a 65-year-old male patient with a 41-year-long history of severe recurrent symptoms a novel insertion, Ains1085 was detected. In a woman with symptoms mainly in the inguinal region a 27-nucleotide-long deletion of the promoter region of the ATP2C1 gene was identified. These mutations did not occur in 50 healthy control patients. Our study further illustrates the diversity of mutational events in the background of HHD.

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New *KIT* Mutations in Patients with Piebaldism

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Piebaldism is a relatively rare, autosomal dominantly inherited disorder, characterized by congenital leukoderma, most commonly involving the forehead, abdomen, and knees. The leukoderma is usually stable throughout life, although pigmented macules may develop at the margins and even within the white macules. Most patients with piebaldism have mutations of the *KIT* gene, which encodes the stem cell growth factor (SCF) receptor, a type III transmembrane receptor tyrosine kinase with an extracellular domain that binds SCF. In the skin, KIT is expressed in mast cells and melanocytes. When activated by ligand binding, KIT stimulates proliferation of melanocytes and is essential for melanocyte development. A mouse model for human piebaldism, ω dominant white spotting, similarly results from mutations involving the murine *Kit* locus. Twenty-one missense mutations, two nonsense mutations, five frameshift mutations, four splice site mutations, one in-frame mutation, and various complete and partial large deletions of the *KIT* gene have been reported in human piebaldism. Here, we describe six novel mutations(142delG, 1768–1769delAG, 2139delC, 2246–2249delAAAG, M541L and Y870C) that result in piebaldism, augmenting our knowledge of genotype-phenotype correlation in this disorder.

147 [Oral 020]

Foxp3 Expressing Regulatory CD4$^+$CD25^{high} T-cells in Human Metastatic Melanoma Lymph Nodes

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Thymic-derived CD4$^+$CD25^{high} regulatory T-cells (T_{reg}) have been recently shown to play a critical role in the immune tolerance and the control of auto-immunity. Since tumor cells express at least partly auto-antigens, T_{reg} might also impair antitumor immune responses directed toward these cells. Indeed, in murine tumor models, tumor rejection is increased upon elimination of T_{reg}. We investigated herein the idea that T_{reg} could be involved in the control of the local immune response in human metastatic melanoma. In a group of 12 patients with stage III melanoma which received no treatment other than surgery, we demonstrated that CD4$^+$CD25^{high} T-cells were overrepresented with a two-fold increased frequency in metastatic lymph nodes, compared to both non metastatic lymph nodes and autologous PBMCs. These tumor infiltrating CD4$^+$CD25^{high} T-cells expressed the Foxp3 transcription factor, which is at this time the only specific marker available for T_{reg}. They displayed a phenotype of activated T-cells (high CTLA-4, CD69 and CD27 levels) and inhibited *in vitro* the proliferation and cytokine production (IL-2, IFNγ) of autologous infiltrating CD4$^+$CD25$^-$ and CD8$^+$ through a mechanism which appears to be dependent on both cell contact and immunomodulatory cytokines (TGFβ, Il-10). Thus, they behave as previously described CD4$^+$CD25^{high} T_{reg}. Infiltrating CD4$^+$CD25^{high} T_{reg} expressed a largely polyclonal TCR repertoire and not a repertoire restricted to a low number of antigenic epitopes. We also found that infiltrating CD4$^+$CD25$^-$ T-cells secreted IL-10 and/or TGFβ and suppressed the proliferation and IFN-γ production of infiltrating CD8$^+$ T-cells, providing evidence that cells with a Tr1/Th3-like phenotype were also present in melanoma metastatic lymph nodes. Moreover, as recently described for murine lymph nodes and as expected, we also found activated CD4$^+$CD25^{high} T-cells displaying some suppressive activity in tumor-free lymph nodes. Altogether, T_{reg} are a major dominant immunosuppressive component of the microenvironment of human metastatic melanoma lymph nodes. These data provide a new basis for the design of future immunotherapeutic strategies aiming to counteract *in vivo* the effects of T_{reg} in patients with melanoma.

148 [Oral 016]

Rab11 is Required for Birbeck Granule Biogenesis

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Birbeck granules (BGs) are organelles specific to Langerhans cells (LCs). This exclusive cellular distribution is dependent on the expression of Langerin, a C type lectin. In a first study we suggested that BGs form where Langerin accumulates, i.e., in the LC tubular endosomal recycling compartment. However, the molecular mechanisms underlying this segregation remain obscure. To identify potential molecular partners of Langerin involved in the biogenesis of BGs, we first reconstituted Langerin traffic and BG formation in the endosomal pathway of the human melanoma cell line M10. In the selected Langerin transfected cell line, M10-Langerin, at the steady state, Langerin mainly concentrated in membranes related to the Rab11$^+$ endosomal recycling compartment. Langerin also recycles in M10-Langerin cells and drives BG biogenesis in the tubular endosomal recycling compartment. We then transfected the M10-Langerin cells with Rab11 wild type and mutants to evaluate ensuing effects on BG formation. Expression of Rab11 constitutively active mutant led to a redistribution of Langerin and of the BGs from the peri-centriolar area towards cell periphery. In contrast, transfection with a dominant negative Rab11 mutant led to the formation of a Langerin positive tubular network, similar to that seen after treatment with Brefeldin A. Importantly, while BGs were still part of the network induced by Brefeldin A, in the absence of an active Rab11 BGs were no longer present. We conclude that Langerin distribution is dependent on Rab11 activity and that the biogenesis of BGs itself is dependent on the presence of an "active" Rab11 molecule, indicating a new role for Rab proteins in organelle biogenesis.

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Efficacy of the Fully Human Monoclonal Antibody MOR102 Against ICAM-1 in the Psoriasis SCID Mouse Model

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Skin-homing memory T lymphocytes play an important role in the pathogenesis of psoriasis by interacting with vascular adhesion molecules and trafficking into lesional skin. Thus an attractive option for targeted therapy of this disease would be blockade of lymphocyte extravasation. We have generated the fully human monoclonal antibody MOR102 against intercellular adhesion molecule 1 (ICAM-1) involved in lymphocyte adhesion to endothelial cells, lymphocyte extravasation and co-stimulation of T cells. MOR102 was selected from the Human Combinatorial Antibody Library (HuCAL$^{®}$) and then converted to the human IgG4 format. The antibody demonstrated efficient inhibition of lymphocyte adhesion to recombinant ICAM-1 with an IC_{50} of $\sim$3 nM. In addition, MOR102 reduced lymphocyte proliferation in mixed lymphocyte cultures by $\sim$50% at concentrations as low as 0.5 μg/ml. Subsequently, the *in vivo* efficacy of MOR102 was tested on grafts derived from lesional skin of three patients with chronic plaque-stage psoriasis transplanted onto SCID mice. Intraperitoneal injection of 10 mg/kg of MOR102 antibody every other day over a period of 4 weeks resulted in reconstitution of orthokeratotic differentiation and a significant ($p < 0.05$) reduction in epidermal thickness as well as marked reduction in the inflammatory infiltrate when compared to PBS treated grafts. The effects observed *in vivo* were comparable to those of dexamethasone (0.2 mg/kg, daily intragastric administration) used as positive control.

Based on the efficacy of the fully human monoclonal antibody MOR102 demonstrated *in vitro* as well as *in vivo* in the psoriasis SCID mouse model, initiation of clinical studies is indicated.

150 [Oral 031]

Induction of Heme Oxygenase-1 (HO-1) Inhibits Dendritic Cell Differentiation and Adaptive Immunity

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The strong immunosuppressive potency of the stress protein HO-1 has been proven in several models of autoimmunity and transplantation. The underlying immune mechanisms, however, are poorly characterised. In our study, the potent HO-1 inducer Cobalt-Protoporphyrine IX (Co-PPIX) strongly suppressed T-cell proliferation in mixed lymphocyte reaction (MLR). As possible mechanism we demonstrated a selective Co-PPIX induced increase of HO-1 expression in monocytes associated with depression of accessory molecule expression and stimulatory cytokine secretion. The likewise induction of HO-1 in monocyte-derived dendritic cells (MDDC) by Co-PPIX was associated with an almost complete inhibition of the differentiation, maturation, and function of MDDC. So, a strong decrease of the expression of DC markers (CD1a, CD83) and accessory molecules (HLA-DR, CD86) was observed. Whereas IL-12 secretion was inhibited, IL-10 production increased. The antigen-presenting capacity of Co-PPIX-treated MDDC was strongly diminished in lymphocyte transformation assay and MLR. The specifity of these effects was demonstrated by HO-1 transduction in immature MDDC. Additional investigations regarding effects of Co-PPIX on genome-wide gene expression and signal transduction pathways in monocytes and DC are ongoing. Together these changes indicated a swith of the DCs to an immature, non-stimulatory phenotype. *In vivo*, Co-PPIX treatment before challenge dose-dependently deressed ear inflammation in DNFB (Type 1) and TMA (Type 2) induced contact dermatitis in mice. Remarkably, Co-PPIX even more strongly inhibited T-cell-dependent inflammation when applied around sensitisation. We hypothesise that the inhibition of DC differentiation, maturation, and function is a crucial mechanism for the suppression of adaptive immunity by HO-1 induction *in vitro* and *in vivo*.

151 [Oral 057]

Relative Contributions of Different Immunocyte Populations to the Development of a Psoriasis Like Inflammatory Skin Disease in Mice with Epidermis Specific Deletion of I Kappa B Kinase 2 Stratis

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Nuclear factor kappa B (NF-κB) proteins play an important role in immune responses and cellular survival. Activation of NF-κB is mediated by the I kappa B kinase (IKK) complex. IKK is composed of the IKK1 and IKK2 catalytic subunits and the regulatory subunit NEMO (NF-κB Essential Modulator). Mice with an epidermis specific deletion of IKK2 (IKK2^{EKO}) exhibit a severe skin disease shortly after birth that is characterised by pronounced acanthosis, hyper- and parakeratosis, intraepidermal pustule formation, dilated blood vessels in the dermis and a dense inflammatory infiltrate consisting of T lymphocytes, macrophages, granulocytes and mast cells in the upper dermis. Macroscopic and histological features of this disease are reminiscent of human psoriasis. Akin to psoriatic skin, epithelium lining macrophages are present in the skin lesions of IKK2^{EKO} mice. The development of the inflammatory response in the skin depends on the presence of the type I receptor for tumor necrosis factor and can be delayed by systemic application of TNF neutralizing proteins. We are currently investigating further pathogenic mechanisms involved in the onset of this skin disease. Using genetic and pharmacological approaches we have selectively eliminated different types of inflammatory cells from the skin of IKK2^{EKO} mice: $\alpha\beta$ T lymphocytes, macrophages and granulocytes. Whereas elimination of $\alpha\beta$ T lymphocytes had no influence on the course of the disease, both elimination of skin macrophages by injection of liposome encapsuled clodronate subcutaneously and inhibition of granulocyte invasion by targeted deletion of the CD18 gene attenuated the inflammatory phenotype, although to different degrees. Comparative analysis of skin changes in the absence of macrophages or granulocytes indicates that skin inflammation in IKK2^{EKO} mice is primarily driven by skin macrophages.

152 [Oral 056]

VLA-1 Dependent Intraepidermal T Cell Expansion is Necessary for the Development of Psoriasis

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Psoriasis is a chronic relapsing T cell-mediated skin disorder. Activated T cells migrating into the epidermis seem to be required for disease formation. Trafficking of leukocytes within tissue and thereby generating an inflammatory response is critically dependent on interactions with extracellular matrix (ECM) proteins such as collagens. We investigated the functional expression of a major collagen-binding receptor: $\alpha_1\beta_1$ integrin (VLA-1) in psoriasis.

VLA-1 was exclusively expressed by infiltrating epidermal but not dermal CD8$^+$ and CD4$^+$ T cells in active psoriasis lesions. To determine the *in vivo* relevance of VLA-1 expression on activated T cells in psoriasis we took advantage of a recently described xenotransplantation model with spontaneous development of psoriasis. Uninvolved human skin from patients with psoriasis was engrafted onto AGR mice, mice deficient in type I and type II interferon receptors in addition to being RAG$^{-/-}$. A time course experiment revealed that the expansion of the epidermal T cell pool correlated well with the onset of a psoriatic phenotype indicating that epidermal T cells are major effectors in psoriasis. *In vitro,* migration of human activated, VLA-1 expressing T cells through collagen IV could significantly be blocked by adding anti VLA-1 antibodies. Therefore we tested the potential of anti VLA-1 treatment to block T cell migration through the basal membrane and thereby to inhibit the expansion of epidermal T cells *in vivo*. Upon transplantation, mice were treated with either murine anti-human α_1-integrin mAb or isotype control antibody. Compared to isotype control, anti VLA-1 treatment led to a significant and almost complete blockade of epidermis infiltrating T cells. Given that epidermal T cells are key effectors we wanted to know if inhibiting the migration of dermal T cells into the epidermis would also block psoriasis development. Skin grafts on mice receiving control antibody developed a fully-fledged psoriasis after 6 weeks as expected. In contrast, blockade of VLA-1 inhibited psoriasis formation in 6 out of 6 mice ($n = 3$ patients). Anti VLA-1 treatment resulted in a significant reduction of both acanthosis and papillomatosis index.

Our findings suggest a crucial role for intraepidermal VLA-1 expressing T cells in psoriasis and might provide the basis for new strategies in psoriasis treatment focusing on T cell/ECM interactions.

153 [Oral 025]

Blockade of Extracellular-Signal-Regulated Kinases 1/2 Deranges Chemokine Expression in Keratinocytes Leading to Enhanced Skin Inflammation

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Prominent epidermal growth factor receptor (EGFR) activation is a common feature of T cell-driven inflammatory skin disorders, and is directly responsible of epidermal hyperplasia and thickening. We have recently demonstrated that EGFR activation also participates in the control of chemokine expression in human keratinocytes, by augmenting IL-8/CXCL8 expression and down-regulating MCP-1/CCL2, RANTES/CCL5 and IP-10/CXCL10. Here we investigated the intracellular mechanisms involved in EGFR-mediated control of TNF-α-driven chemokine expression. When associated to TNF-α, exogenous TGF-α efficiently induced a persistent potentiation of Extracellular-Signal-Regulated Kinases (ERK)1/2 and a modest, transient increase in JNK and p38 MAPK activity. By contrast, EGFR functional blockade by specific inhibitors prevented ERK1/2 activation completely, without relevant effects on JNKs and p38 MAPKs. Selective inhibition of either EGFR or ERK produced a similar decrease of IL-8 and increase of MCP-1, RANTES and IP-10 expression. Reporter gene analysis demonstrated that both the inhibitors strongly suppressed TNF-α-driven IL-8 promoter activity, whereas they did not affect the transactivation of MCP-1 promoter. These results suggested that ERK1/2 was directly involved in the control of the transcription of IL-8, but not of MCP-1 gene in human keratinocytes. Also, they indicated that the increased expression of MCP-1 mRNA following EGFR or ERK1/2 inhibition could rather depend on post-transcriptional events. Indeed, mRNA decay kinetics performed in the presence of mRNA synthesis inhibitors demonstrated that EGFR or ERK1/2 blockade was associated to prominent stabilization of MCP-1 mRNA. Finally, skin application of a selective ERK1/2 inhibitor led to worsening of both irritative and allergic contact dermatitis in mice, with significant increase of edema and macrophage/monocyte infiltration in both the dermis and epidermis. In conclusion, ERK1/2 activation appears to mediate important anti-inflammatory functions in the skin. Its inactivation causes enhanced expression of several potent epidermal chemokines, which potently attract leukocytes and lead to enhanced skin inflammation.

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Identification of Toll-like Receptors and Characterisation of the Signal Transduction Pathway During *Candida albicans* Infections

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Recognition of the human pathogenic fungus *Candida albicans* by the innate immune system is the first approach to activate a rapid immunological response. It takes place by Toll-like receptors (TLRs) which are specialized for recognition and binding of evolutionary conserved pathogen associated molecular patterns (PAMPs). Activation of TLRs upon PAMP binding leads to the induction of transcription factors such as nuclear factor (NF)-κB, and to a subsequent inflammatory response which is crucial for the successful defeat of candidosis.

Here we analyzed the role of Toll-like receptors (TLRs) by using TLR2, TLR4, TLR2/4-deficient murine macrophages, respectively, and human embryonic kidney (HEK) 293 cells transfected with human TLR2 or TLR4-expression plasmids upon stimulation with two *C. albicans* preparations, viable and antimycotics (AM)-treated *C. albicans*. We found that both TLR2 and TLR4 were involved in recognition of viable *C. albicans* wild-type strains, whereas *C. albicans* treated with a mixture of three antifungal drugs (i.e. amphotericin B, nystatin, and itraconazole) mainly employed TLR2 in murine macrophages and transfected HEK293 cells. In both cell types, preincubation of macrophages with the stimulating cytokine interferon (IFN)-γ led to the ability of TLR2 to sense viable *C. albicans* cells in the absence of TLR4, while AM-treated *C. albicans* still were sensed by TLR2 in a TLR4- and IFN-γ-independent manner. The differences in the TLR activation pattern of viable *C. albicans* as compared to *C. albicans* treated with cytoplasmic membrane-interacting antimycotics suggest specific recognition of different cell wall PAMPs by TLRs in innate antifungal responses.

Analysis of the signal transduction cascades following viable *C. albicans* recognition demonstrated phosphorylation and degradation of IκB-α, the inhibitory subunit of NF-κB. Moreover we could show, that c-Jun NH_2-terminal kinase (JNK) and c-Jun, a component of the transcription factor AP-1, as well as the mitogen activated protein (MAP) kinases p38 and p44/42 were activated. Our work shows that in addition to NF-κB, AP-1 is involved in the cellular response to *C. albicans* and points towards a cross-talk between these transcription factors which enables the host to fine-tune the innate immune response to fungal challenges.

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***Pseudomonas aeruginosa* Protease IV Induces Proinflammatory Mediators and Antibiotic Peptides in Keratinocytes**

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Intact human epidermis is rarely colonized by *Pseudomonas aeruginosa.* Culture supernatants of this gram-negative bacterium are supposed to contain pathogen-associated molecules responsible for the induction of expression of proinflammatory cytokines and antimicrobial peptides in human epithelia. Several substances are released by the organism into the environment. Among them bacterial exoproteases are good candidates for the stimulation of cellular activities in keratinocytes. Culture supernatants of *P. aeruginosa* strain ATCC 11440 were partially purified by sequential ultrafiltration in steps of 300 kDa, 30 kDa and 3 kDa. Investigation of the 30–300 kDa fraction revealed a trypsin-like enzymatic activity and calcium-mobilizing activity in HaCaT keratinocytes. Further purification by p-amino-benzamidine affinity chromatography led to a compound with lysine-specific proteolytic activity with steady calcium-mobilizing activity in keratinocytes. The specific cleavage of tosyl-glycyl-prolyl-lysine-pNA, the typical inhibitor profile and detection in SDS-PAGE proved the identity to protease IV from *P. aeruginosa.* It could be shown by single cell fluorescence imaging microscopy, that the purified enzyme induced calcium-influx in HaCaT keratinocytes as well as in primary keratinocytes with similar time course and potency as compared with trypsin and thrombin. Pre-stimulation of HaCaT keratinocytes with thrombin and trypsin abolished a subsequent response to protease IV. This points towards a possible involvement of protease-activated receptors on keratinocytes. RT-PCR and real-time-PCR analysis showed that protease IV induced pronounced mRNA-expression of the proinflammatory cytokines IL1β, IL-8 and TNFα and the antimicrobial peptides HBD-2 and RNase-7 in HaCaT keratinocytes. Protease IV could be defined as a pathogen-associated molecule which is secreted into the environment. By utilization of a calcium-dependent signalling pathway the enzyme induces epidermal defence mechanisms such as cytokine and antimicrobial peptide expression in human keratinocytes. Thus, protease IV seems to be involved in the recognition of cutaneous *P. aeruginosa* colonization by keratinocytes followed by the initiation of innate immune responses.

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Depletion of Human CD4$^+$ T Cells in Peripheral Blood and Skin by HuMax-CD4

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CD4$^+$ T cells are involved in inflammatory skin diseases and are the malignant T cells in the majority of Cutaneous T cell Lymphomas. Targeting CD4 may, therefore, inhibit or eliminate disease-driving T cells, which could be a useful approach in the treatment of inflammatory and malignant T cell driven skin diseases. In this study we used HuMax-CD4, which is a fully human antibody against CD4. First, 20 human volunteers were treated with either placebo, or one of four doses of HuMax-CD4 (0.5, 1, 2, or 4 mg/kg) iv. once. HuMax-CD4 treatment resulted in a dose-dependent decrease in the number of circulating CD4$^+$ T cells with maximum at 4 mg/kg 12 hours following injection (placebo: −3.5 (−27; 22); HuMax-CD4: −75 (−83; −19) (median (range) of change in percent from baseline)). Then we investigated whether HuMax-CD4 depleted skin infiltrating CD4$^+$ T cells in a human inflammatory skin disease model, the human psoriasis SCID mouse xenograft model. HuMax-CD4 treatment resulted in a significant reduction in numbers of skin infiltrating CD3$^+$ T cells (PBS: 39.9 ± 16.4 (n = 8); HuMax-CD4: 4.7 ± 0.9 (n = 7); $P = 0.001$ (mean ± SEM)). The reduction in CD3$^+$ T cells was not due to a reduction in the subgroup of CD8$^+$ T cells (PBS (n = 8): 15.5 ± 6.5; HuMax-CD4 (n = 7): 12.6 ± 4.6; $P = 0.82$ (mean ± SEM)), but due to a significant decrease in the CD4$^+$ T cells measured by a non-competing CD4 antibody (NCL-CD4-368) (PBS (n = 8): 18.2 ± 5.9; HuMax-CD4 (n = 7): 3.0 ± 1.0; $P = 0.003$ (mean ± SEM)). Mechanisms behind HuMax-CD4's ability to deplete T cells were investigated *in vitro*. HuMax-CD4 did not induce complement-dependent cytotoxicity or apoptosis of CD4+ T cells but was highly effective in triggering antibody-dependent cell-mediated cytotoxicity with natural killer cells as effectors (14–90% specific lysis (n = 10)). Furthermore, HuMax-CD4 inhibited T cell activation and proliferation in a dose-dependent manner. The ability of HuMax-CD4 to deplete CD4$^+$ T cells in peripheral blood and skin makes the antibody a candidate for therapy of inflammatory and malignant skin diseases involving CD4$^+$ T cells.

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Generation of Regulatory T cells by Retroviral Gene Transfer of *Foxp3*

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Immunological unresponsiveness to self is mediated in part by regulatory CD4$^+$CD25$^+$ T cells (Treg), which can actively suppress self-reactive T cells that have escaped thymic selection. The isolation of large numbers of Treg is difficult, since CD4$^+$CD25$^+$ cells constitute only a small subset of CD4$^+$ T cells. Recently, the transcription factor *Foxp3* has been identified to be a key regulatory gene for the development of Treg. The *Foxp3* cDNA was cloned into a MCSV-based retroviral vector encoding eGFP under control of an IRES site and used to transfect the GPE86 packaging cell line. For controls a retrovirus containing only eGFP was employed. Retrovirus-containing culture supernatants were utilized for *Foxp3* gene transfer into murine naive CD4$^+$CD25$^-$ T cells to investigate if these cells would develop a regulatory phenotype. Control virus-transfected T cells proliferated, whereas *Foxp3* transfected T cells were anergic. Flow cytometry analysis of *Foxp3* transfected T cells revealed expression of CD25, CTLA-4, and neuropilin-1, all molecular markers associated with Treg. Subsequently, GFP$^+$ T cells were added to CD4$^+$CD25$^-$ T cells to study their suppressor function. Naive CD4$^+$CD25$^-$ cells that were control virus-treated failed to inhibit T cell proliferation. In contrast, naive T cells that had been transfected with the *Foxp3* containing retrovirus showed strong suppressor activity. A mechanism by which Treg suppress other T cells is the production of IL-10. Only *Foxp3* gene-transfected T cells but no control virus-treated T cells expressed IL-10. We are currently using *Foxp3* transfected T cells for the treatment of systemic autoimmunity in K14-CD40L transgenic mice in order to ameliorate disease. Together, gene transfer of *Foxp3* converts naive T cells towards a regulatory phenotype similar to that of natural Treg. Furthermore, *Foxp3* gene transfer may be useful to generate large numbers of Treg for immunotherapy of autoimmune disorders.

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Immunization with Peptide or Protein Onto Ultraviolet Irradiated Skin to Induce Antigen-Specific Cd8$^+$ T Cell Unresponsiveness

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In a murine model, repeated cutaneous exposure to low doses of ultraviolet B (UVB) impairs the induction of contact hypersensitivity response to haptens and induces hapten-specific suppressor T lymphocytes. We investigated whether UVB can be used to induce antigen-specific unresponsiveness to protein or peptide antigens. Using an adoptive transfer model, we have determined that UVB induces *in vivo* suppression of antigen-specific CD8$^+$ T cell proliferation, IFNγ production and cytotoxic capability in response to ovalbumin (OVA) protein immunization in C57BL/6 mice. This effect is strictly IL-10 dependent as no UVB suppression of CD8$^+$ T cell responses to protein antigen is noted in IL-10 deficient mice. Immunization on UV irradiated skin further induces antigen –specific CD4$^+$ regulatory cells since transfer of CD4$^+$ T cells from UVB irradiated and OVA-immunized but not control protein –immunized mice suppresses the CD8$^+$ T cell response to OVA immunization in non-irradiated mice. Phenotypic analysis of these CD4$^+$ T cells reveals that the suppressor effect of the CD4$^+$/CD25$^+$ T cell subset is dominant and depletion of this subset abrogates the adoptive transfer of suppression. Further, immunization of UVB irradiated mice with an I-A^b –restricted OVA peptide, induces the generation of regulatory T cells that prevent the priming of CD8$^+$ T cells to full OVA protein. Epidermal sheet staining of mouse skin after UV irradiation shows depletion of I-A$^+$ Langerhans cells in the epidermis. In contrast to the effect on immunization with protein antigen, UVB failed to induce suppression of CD8$^+$ T cell priming in mice immunized with the K^b- restricted OVA peptide, SIINFEKL. We suggest that UVB alters the functional maturation of cutaneous dendritic cells resulting in the induction of CD4$^+$/CD25$^+$ regulatory T cells that inhibit antigen-specific CD8$^+$ T cell priming and in the generation of APC that fail to prime CD8$^+$ T cells to protein antigen. The ability to generate antigen-specific suppression using UVB to limit CD8$^+$ T cell responses may be of therapeutic value to prevent CD8$^+$ T cell mediated auto-immune disease and transplantation rejection.

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Human Beta-Defensin-2 Induction in Keratinocytes is Controlled by Nuclear Factor-κB Family and Activating Protein-1

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Human beta-Defensin-2 (hBD-2) is an inducible peptide antibiotic and part of the innate chemical defence barrier of human skin which controls the physiological flora and protects us in a very effective, fast responding way against invading microorganisms. The expression of hBD-2 in keratinocytes is induced by proinflammatory cytokines and bacteria, especially by IL-1β and *Pseudomonas aeruginosa* (PA). Since little is known about the signal transduction pathways leading to hBD-2 gene induction, we investigated the transcription factors involved in IL-1β and PA-mediated gene induction of hBD-2. We transfected HaCaT keratinocytes with mutation constructs of hBD-2 promoter-driven luciferase reporter plasmids. Stimulation was performed using supernatants of mucoid PA or IL-1β. The mutation of three potential NF-κB binding sites resulted in a strongly diminished induction of promoter activity with the mutation of the binding site next to exon-1 being the most reducing. Mutation of a potential AP-1 binding site reduced promoter activation by more than 70%. A combination of all four mutations completely abolished hBD-2 promoter activity following IL-1β or PA treatment. To further analyse the influence of NF-κB and AP-1 on hBD-2 gene induction, we investigated the effect of the NF-κB inhibitor Helenalin and inhibitors of the three AP-1 inducing MAP-kinase cascades (ERK1/2 pathway, JNK pathway and p38 pathway). Realtime PCR and ELISA studies following stimulation of HaCaT and primary keratinocytes with PA or IL-1β and co-incubation with the specific pathway inhibitors confirmed the involvement of NF-κB and AP-1: the specific NF-κB-inhibitor Helenalin almost completely abolished hBD-2 induction; the JNK-Inhibitor SP600125 suppressed hBD-2 induction by approximately 60%; the p38 MAP-kinase inhibitor SB202190 diminished hBD-2 induction by approximately 50%, but ERK1/2-inhibitor PD98059 showed no modulating effect on PA or IL-1β-mediated hBD-2 induction. To determine the NF-κB subunits, responsible for hBD-2 induction, we established a transcription-factor ELISA: nuclear extracts of PA or IL-1β treated primary keratinocytes were incubated with oligonucleotides matching the NF-κB sequence of hBD-2 promoter. Subsequently binding of NF-κB subunits was analysed using specific antibodies. These studies revealed an increased binding of the NF-κB family members p65 (RelA) and p50 to the NF-κB consensus sequence next to exon-1 of the hBD-2 gene. Binding of NF-κB subunits p52, RelB and C-Rel was not affected. Our data indicate that the NF-κB family, especially subunits p65 (RelA) and p50, is essential for hBD-2 induction following IL-1β or PA stimulation. In addition to NF-κB, AP-1 also seems to be involved in IL-1ß or PA mediated hBD-2 induction following activation of the JNK and p38 pathway. We conclude that the synergistic activation of NF-κB and AP-1 is essentially required for fully IL-1β or PA mediated hBD-2 promoter activation in human keratinocytes.

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Analysis of Cutaneous Immune Surveillance in Transgenic Mice Expressing Ova in the Skin

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Cutaneous immune surveillance seems to be important in host defence mechanisms. However, the surveillance function of the skin has never been definitively proven to be operative *in vivo* under physiological conditions. Depending on the cellular and molecular microenvironment, immune responses generated by skin associated lymphoid tissues can lead not only to protective immunity against pathogens, but also to tolerance.

To study immune responses to a de novo expressed antigen in the skin under homeostatic conditions *in vivo*, we have generated transgenic mice expressing the surrogate antigen ovalbumin (OVA) in a Cre-inducible manner in keratinocytes (K5-loxP-YFP-loxP-OVA-IRES-CFP). Expression of OVA is either induced around embryonic day 14.5 with a constitutively expressed K5-Cre transgenic line or in adult mice with an inducible K5-CreTM transgenic line after tamoxifen administration. Several transgenic lines have been obtained, which, in an uninduced state, express the yellow fluorescent reporter gene (YFP) but not the OVA gene in epidermal cells. After Cre-mediated excision of YFP, OVA can be induced, as evidenced by the expression of the $OVA_{257-264}$ peptide SIINFEKL on keratinocytes by FACS analysis. Moreover, these keratinocytes are able to stimulate the TCR_{OVA} specific T-cell hybridoma cell line 4B10 to produce IL-2. Although OVA is efficiently expressed and presented, the transgenic mice do not develop any obvious disease symptoms like hair or weight loss. Moreover, no differences in thymic and peripheral T-cell numbers can be observed after Cre-mediated OVA expression. After immunization with OVA and complete Freund's adjuvant there is no significant difference in ear swelling compared to controls. To monitor CD8+ T-cell mediated immune responses the transgenic mice were crossed with OT1 mice expressing a TCR specific for SIINFEKL-$H2k^b$ on all T-cells. No differences in the numbers of antigen specific T-cells could be seen in the periphery, although we find activation of antigen specific T-cells as indicated by upregulation of CD44.

Taken together our results suggest that expression of a de novo antigen in the skin and thymus of newborn and/or adult mice does not affect the development and reactivity of antigen specific T-cells. We are currently investigating whether an additional danger signal is required to get an anti-OVA-specific immune response. Moreover, we are also comparing the effects of peripheral (keratinocytes) versus central (DC) OVA expression in transgenic mice expressing a Cre-inducible OVA from the CD11c promoter in DCs.

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Relevance of β2-integrins in the Reverse Arthus Reaction

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The Arthus reaction is a classical experimental model of type III hypersensitivity, characterized by leukocytoclastic vasculitis of small blood vessels due to deposition of immune complexes. The consequent tissue injury is mediated by inflammatory cell infiltration that is highly regulated by various adhesion molecules. β2-Integrins are of critical importance for leukocyte recruitment and extravasation through vascular endothelia. However, the specific roles of these molecules in hypersensitivity vasculitis have not been clearly defined.

To assess the contribution of integrins in this pathogenic process, the cutaneous reversed passive Arthus reaction was examined in mice lacking the β2-integrin subunit, CD18. Edema and haemorrhage were severely suppressed and tissue damage, revealed by extravasation of fluorescent-labelled bovine serum albumin, was significantly reduced in CD18(−/−) mice compared with controls. Histological examinations showed the typical picture of leukocytoclastic vasculitis only in CD18(+/+) mice, while CD18(−/−) mice showed low number of infiltrating leukocytes, and no signs of vasculitis. Functionally, we found no differences between CD18(+/+) and CD18(−/−) granulocytes in their capacities of degranulation and producing oxidative burst when bound to immune complexes. The inhibited vasculitic reaction could be restored after injecting CD18(+/+) viable granulocytes in CD18(−/−) mice intravenously, but not after subcutaneous administration. This suggests that, although immune-complex deposition occurs at both sides of the vessel wall, the damaging processes take place only via immune complexes deposited at the luminal site, during diapedesis of leukocytes. However, deposition of immune complexes alone is not sufficient to produce leukocytoclastic vasculitis. Even though the capacity of granulocytes to degranulate is not altered in CD18(−/−) mice and, consequently, CD18 is not directly involved in immune complex-mediated endothelial damage, absence of CD18 sufficiently prevents granulocytes from exerting their deleterious effect on vessel walls. This is due to impaired recruitment of granulocytes in the skin in CD18 deficiency.

We here dissect basic mechanisms also underlying the therapeutical blocking of CD18 in vasculitis and autoimmunity.

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Identification, Isolation and Characterization of the Release Kinetics of a Distinct Rantes-Containing Secretory Granule System In Human Lymphocytes – Relevance for the Atopy Syndrome

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The beta-chemokine RANTES is stored in granules of platelets and eosinophilic granulocytes. We now describe the discovery, characterization, isolation and release kinetics of a unique pan-lymphocytic RANTES-granule system. Flow cytometry, laser scan microscopy, ELISA, sucrose gradients, western blotting, activation of cells using various stimuli and various inhibitors was employed. These RANTES-granules did not colocalize with known markers of endosomes, lysosomes, secretory lysosomes or the MHCII-compartment. They were of different density than granzyme-B contaning granules and could be mobilized to the cell surface within one hour after an appropriate secregous stimulus like a combination of phorbol ester and calcium ionophore, and/or anti-CD3 crosslinking in the case of T cells. The release into the supernatant was independent of protein neosynthesis and was significantly faster as known secretory organelles of lymphocytes like lytic granules of cytotoxic T lymphocytes or natural killer cells. Investigating the peripheral blood from atopic patients, where RANTES is supposed to play an important role in pathophysiology, their RANTES-granule system was found to be significantly altered as compared to healthy individuals, namely a RANTES-hyperreleasability is described. Taken together, a new secretory cell organelle in lymphocytes is described which is stuffed with RANTES. The discovery of a RANTES-hyperreleasability in atopic patients supports the idea of a cell type independent defect of secretory granules as a major factor in the pathogenesis of atopy syndrome.

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Can Deletion Of Autoreactive T Cells Protect From Autoimmune Pancreatitis?

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It is widely accepted, that organ-specific autoimmune diseases, such as multiple sclerosis, psoriasis, rheumatoid arthritis and autoimmune diabetes are mediated by interferon (IFN-)γ producing Th1 cells targeting organ-specific antigens. Yet, recent models of autoimmune diabetes suggest that beta cell destruction can proceed in the absence of beta cell surface antigen recognition by specific T cells. To investigate the role of autoreactive Th1 cells in organ-specific autoimmune disease and the protection provided by clonal deletion, we used a mouse model of autoimmune pancreatitis. 415-EL-I-E C57BL/6 mice express MHC II I-E exclusively on the exocrine pancreas. T cell reactivity to this 'non-self' MHC class II I-E antigen is associated with T cell receptor containing vβ5 chain and in 415-EL-I-E mice 4% of the $CD4^+$ T cells express the vβ5 chain. In these mice TCR-$v\beta5^+$ $CD4^+$ T cells are not clonally deleted and proliferation as well as IFN-γ production of $v\beta5^+CD4^+$ T cells was normal in 415-EL-I-E C57BL/6 mice. Histological analysis of the pancreas showed inflammation in 50% and destruction in another 40% of 415-EL-I-E mice. To prevent autoimmune pancreatitis we crossed 415-EL-I-E mice to 107-I-E mice, expressing MHC class II I-E on B cells, macrophages, dendritic cells and thymic epithelium. In 415-EL-I-E × 107-I-E F1 mice $v\beta5^+$ T cells were decreased up to ten fold and did not proliferate in response to mAb. Yet, deletion of autoreactive $v\beta5^+$ T cells did not prevent pancreas destruction in 415-EL-I-E × 107-I-E mice. To study the role of cross-reactive T cells we further crossed 415-EL-I-E mice to immune deficient C57BL/6 RAG-$2^{-/-}$ mice. Flow-cytometric analysis of the lymphatic tissues of 415-EL-I-E × RAG-$2^{-/-}$ mice showed complete absence of T cells. Surprisingly, pancreas destruction in 415-EL-I-E × RAG-$2^{-/-}$ mice was even enhanced. All mice had completely destroyed pancreata. We conclude that autoimmune organ destruction can develop in the absolute absence of T cells. The data raise the question of whether autoreactive T cells are really harmful, or whether they can protect against inflammatory autoimmune disease, at least under certain conditions, where organ destruction may be caused by immune cells like macrophages or NK cells.

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JAM-B and JAM-C are Required For Development Of Cutaneous Inflammation

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A hallmark of inflammation is leukocyte extravasation, which is mediated by adhesion molecules expressed on leukocytes and endothelial cells in a stepwise process. In contrast to the first steps (rolling, activation and adhesion), molecular mechanisms of leukocyte transendothelial migration are less understood. Recently, much attention has been attributed to junctional adhesion molecules (JAMs) in leukocyte transmigration. JAMs are adhesion molecules of the Ig gene superfamily. JAM-A is located at intercellular junctions of endothelial cells and is ligand for the β2-integrinLFA-1. JAM-B and -C are also expressed by endothelial cells and bind VLA-4 or Mac-1, respectively. Since JAM-A antibodies were shown to inhibit leukocyte migration to the skin, we sought to assess if JAM-B and -C contribute to recruitment of leukocytes in cutaneous inflammation. For this purpose mice were sensitized and challenged with DNFB. After development of delayed-type hypersensitivity (DTH, 24 h) ears were snap frozen. Immunofluorescent quantitation of JAM-B and -C expression in histological sections showed a constitutive expression in numerous vessels. No further increase could be observed in DNFB- vs. vehicle-treated ears. However, this does not rule out an involvement of JAM-B and -C in cutaneous inflammation, since at least JAM-A shows a phosphorylation-depended binding and translocation from intercellular junctions to the apical side of the endothelium following an inflammatory stimulus. A blockade of JAM-B or -C 1 hour before DNFB-challenge with blocking antibodies lead to a significant decrease in ear swelling (control Ab: 20.9, anti JAM-B: 4.1, anti-JAM-C 7.1 $mm \times 10^{-3}$; n = 8/group). In conclusion, JAM-B and -C are constitutively expressed in murine vasculature and their expression pattern seems unchanged upon inflammation. Blocking of JAM-B or JAM-C inhibits development of a DTH reaction in mice. Hence, JAM-B and JAM-C may be involved in the pathogenesis of chronic-inflammatory skin diseases, such as psoriasis or atopic dermatitis.

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Mature, but not Immature Plasmacytoid Dendritic Cells Induce Hapten-Specific T-Cell Proliferation

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Plasmacytoid dendritic cells (pDC) are known to play a critical role in the innate and adaptive immune response against viruses. We have recently detected these cells in the epidermal and dermal infiltrate of allergic contact dermatitis elicited by epicutaneous patch tests (EPT). In the dermis they represented a substantial proportion of the entire DC infiltrate and were in close apposition to T-cells, suggesting interactions between pDC and infiltrating T-cells. Further phenotypic analysis revealed that approximately 50% of pDC in EPT lesions express the maturation markers CD83 and CD86.

In this study, we investigated the capacity of immature and mature pDC to stimulate hapten specific T-cells. As a positive control, pDC were compared to $CD1c^+$ myeloid dendritic cells (mDC), which represent the major dendritic cell population in EPT lesions. Nickel-specific $CD4^+$ T-cell lines from nickel-allergic individuals were co-cultured with autologous blood-derived pDC and mDC for 72 h. Before co-culture, both pDC and mDC were exposed to nickel and either left immature or matured by using TNF-α and IL-3 and TNF-α and GM-CSF, respectively.

Nickel-pulsed mature, but not immature mDC acted as strong stimulators of hapten-specific T-cells, thus confirming previous observations. Importantly, nickel-pulsed mature pDC induced a nickel-specific T-cell response comparable in magnitude to that elicited by mDC. As expected, neither unpulsed mature nor immature pDC and mDC were able to stimulate hapten-specific T-cells.

In conclusion, following exposure to nickel, mature pDC acquire the capacity of inducing a hapten-specific T-cell proliferation, indicating that they may act as prominent amplifiers of the adaptive immune response in allergic contact dermatitis. Whether the inability of immature pDC to evoke a proliferative T-cell response reflects their potential to induce T-cell unresponsiveness or even anergy remains to be determined.

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CD1e Undergoes a Double Processing in Endosomes: An N-Terminal Propeptide is Cleaved in Concomitantly to the Conversion of Membrane Associated CD1e into a Soluble Molecule

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The alignment of the cDNA-deduced aminoacid sequences of CD1e from several species suggests that CD1e includes an N-terminal extension of 9–12 aminoacids, as compared to other CD1 molecules. Partial protein data about sheep suggest that endosomal CD1e does not include such an extension. This raises the question as whether CD1e includes an N-terminal extension. A plasma membrane targeted CD1e form was expressed in mammalian transfected cells, as well as a CD1e-eGFP fusion protein, which was localized and cleaved in endosomes, as natural CD1e. Plasma membrane-associated and soluble endosomal CD1e were purified from both types of transfectants. Their N-terminal sequences showed that the membrane-associated CD1e includes an N-terminal extension, which was absent on endosomal soluble CD1e. Presence of the N-terminal extension on neosynthetized CD1e was confirmed using antibodies rose against a synthetic peptide. To define the cellular compartment where the N-terminal end of CD1e is processed, we compared the biosynthesis of recombinant complete and a propeptide-free CD1e. Biochemical experiments showed that the propeptide is cleaved after the Golgi compartment, in a bafilomycin sensitive compartment, quite concomitantly with the cleavage of CD1e into a soluble form. Additional experiments using a non cleavable mutated CD1e suggested that the cleavage of the propeptide and the solubilization of CD1e occurs in substructures of the endosomal compartments. These data demonstrate that CD1e has the unique property of undergoing a double processing in late endosomes. Such a pro-protein structure has implications on the function of CD1e.

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Anti-SC5 a Unique Monoclonal Antibody Reactive to Vimentin Expressed on Cell Surface Membrane of Sezary Cells

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Cutaneous T cell lymphoma (CTCL) represents a heterogeneous group of malignant mature T cell proliferations. The most common forms are the mycosis fungoides and its leukemic counterpart, the Sezary syndrome (SS). Until now, only few cell surface markers are available to identify circulating Sezary cells. Recently, we have raised a monoclonal antibody (mAb) against a molecule named SC5, present on the extracellular membrane and in the cytoplasmic part of Sezary cells. Cell surface staining revealed that $CD4^+SC5^+$ circulating lymphocytes in SS patients are significantly increased in comparison with normal individuals and are correlated with the percentages of morphologically detected Sezary cells in peripheral blood. We have identified the SC5 molecule as the intermediate filaments vimentin. This unexpected cell surface expression of cytoskeleton molecule is related to its transport to surface membrane in early response to activation and its secretion into the extracellular space. Vimentin has been clearly localized on surface of intact fresh Sezary cells and Sezary cell lines by confocal microscopy and flow cytometry analysis. We have also shown seroreactivity against vimentin in some patients with Sezary syndrome and transformed mycosis fungoides. Binding assays and immunoprecipitation assays have shown that SC5 is associated with actin, leucocyte specific protein 1 (LSP1), and α-actinin 4 in Sezary cells and activated lymphocytes. Vimentin is a phosphorylated protein on serine and threonin, which is involved in several signalling pathways and cellular regulation. Thus vimentin cell surface expression suggests a cytoskeletal reorganization that may influence many structural processes like cell morphology, cell adhesion or migration and also proliferation. SC5 mAb could be a novel tool to better understand molecular pathogenesis of the malignancy. Finally SC5 mAb should offer a better identification of circulating malignant CD4 cells, and so improvements of patients follow up.

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Are they Different? Langerhans Cells in the Steady State *Versus* Inflammation

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Migrating Langerhans cells (LC) may induce immunity or – in the steady state – maintain peripheral tolerance. A contact allergy model was employed to investigate changes in migrating LC in steady state versus inflammation. We applied 2,4,6-trinitro-1-chlorobenzene (TNCB) onto skin to induce migration of LC to lymph nodes. Dendritic cells (DC) isolated from skin-draining lymph nodes of TNCB-treated and control mice were compared in phenotype and function. In response to TNCB increased numbers of LC, as determined by anti-Langerin/CD207 staining, appeared in the nodes. Nearly all of the LC already expressed CD80 and CD86 in the steady state, the other members if the B7 family were found just on part of the cells. After induction of inflammation more CD40-, B7-H1 (PD-L1)-, and B7-DC (PD-L2)-positive LC were found in the lymph nodes. Furthermore, levels of costimulatory molecules on LC were slightly enhanced in inflammation. Another B7 family member possibly involved in tolerance induction, ICOS-L, was never detected on LC. When isolated lymph node DC were stimulated with LPS- and anti-CD40 mAb the LC within this population produced IL-12p40 which was increased significantly during inflammation. The augmented IL-12 production correlated with more IFNγ being produced by T cells in lymph nodes draining inflamed skin. After epicutaneous application of ovalbumin protein lymph node DC/LC presented peptides on MHC II to antigen-specific $CD4+$ T cells *in vitro* and *in vivo*. Furthermore, after the induction of inflammation the proliferation of the $CD4+$ T cells was strongly enhanced. Thus, phenotypical differences between LC in steady state and inflammation may not be the predominant markers for immunogenic versus tolerogenic LC. However, cytokine profiles differ strongly and may be a decisive factor for the immunogenicity of LC.

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Study of Constitutive Expression, Cytokinic Modulation and Functional Capacity of Toll-like Receptors (TLRs) in Normal Human Epidermis. Upregulation in Psoriatic Skin

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Recent data gave evidence that TLR play a key role in both innate and adaptive immune response. The human skin is consistently exposed to a wide range of exogenous stimuli and can exhibit anti-infectious properties. The aim of the present work was to investigate the expression of TLRs in human epidermis, to study its modulation by proinflammatory cytokines and to characterize the function of these receptors within the epidermis in physiological and pathological conditions. Primary cultures of keratinocytes established from normal skin biopsies and an epithelial cell line (NCTC 2544) were studied for the expression of TLRs by semi-quantitative RT-PCR, immuno-blot, immuno-cytochemistry and flow cytometry. Alteration in TLR basal expression was quantified after cell exposure to various concentrations (1 to 1000 U/ml) of cytokines (IFNgamma, TNFalpha) during 24 to 72 h. TLR function was studied in the presence of pathogen-specific molecular patterns [PAMPs: peptidoglycans (PGN) for TLR2, double stranded RNA poly (I:C) for TLR3, lipopolysaccharide (LPS) for TLR4] and quantification of NF-kappa B translocation to the nucleus was measured by nuclear DNA-binding activity by electrophoretic mobility shift assay (EMSA) or reporter gene analysis after transfection of NF-κB gene reporter. The production of NF-κB-related cytokines (TNFalpha and IL-8) by keratinocytes was measured in response to TLR stimulation. TLR expression was analysed by immunohistochemistry on biopsies from normal human skin and psoriatic lesions. Our results demonstrate that a large repertoire of TLRs is expressed in normal skin as shown by RT-PCR. Immunohistochemical analysis of skin section showed an expression of TLR3 and TLR4 in the cytoplasm of basal keratinocytes predominantly and TLR2 staining was observed throughout the epidermis. By flow cytometry analysis, human cultured keratinocytes and NCTC 2544 cells were shown to express TLRs with an intense intracytoplasmic localization contrasting with a low level surface expression of TLR2, TLR3 and TLR4. Exposure of keratinocytes to IFNgamma increased intracytoplasmic expression of TLR4 without affecting ectopic expression, whereas TNFalpha stimulated a partial translocation of TLR2 to the cell surface. Stimulation of keratinocytes by PGN, poly (I:C) and LPS led to the nuclear translocation of NF-κB as demonstrated by EMSA and luciferase assay. Moreover, TLR stimulation induced the release of IL-8 and TNFalpha by keratinocytes. The histological comparison of normal and psoriatic skin showed that TLR2 was strongly upregulated in lesional psoriatic skin. In conclusion, we demonstrated that human keratinocytes express a large repertoire of TLRs and functional TLR 2, 3 et 4. These results suggest that the triggering of TLR2 by specific PAMS, *i.d.* PGN from Gram-positive bacteria, is involved in the pathogenesis of psoriasis through the activation of a pro-inflammatory program.

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TRANCE and its receptors TRANCE-R and OPG are Expressed in Epidermis: Implications for Langerhans Cell Longevity

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Recent data indicate that epidermal Langerhans cells (LC) have a marked capacity to survive for long periods of time in the periphery. A newly described TNF superfamily signalling pathway consisting of TRANCE, a decoy receptor, osteoprotegerin (OPG), and a functional receptor, TRANCE-R (also known as RANK) has been associated with numerous activities including regulation of dendritic cell (DC) function. We and others have shown that TRANCE-TRANCE-R interactions support DC survival, allowing prolonged persistence of TRANCE-treated DC in draining lymph nodes as well as extending survival of interstitial DC involved in antigen surveillance. We hypothesised that this system might be of relevance to the prolonged survival ability of LC and in this study sought to define expression of TRANCE and its receptors in epidermis.

mRNA was prepared from normal human skin ($n=3$) and from second passage keratinocyte cultures ($n=3$) and examined by semi-quantitative RT-PCR using primers specific for TRANCE, TRANCE-R and OPG following normalisation with an epidermal housekeeping gene. These studies demonstrated the presence of mRNA encoding TRANCE and both receptors both *in vivo* and in the *in vitro* keratinocyte cultures. We then sought to define the presence of soluble TRANCE protein by ELISA in keratinocyte cultures and TRANCE-R by immunofluoresence of human skin. High levels of soluble TRANCE were detected in 24 hr keratinocyte culture supernatants *in vitro. In vivo*, TRANCE-R was strongly expressed by dendritic cells in the epidermis and in the superficial dermis. Double labelling studies demonstrated that all $CD1a+$ve epidermal and superficial dermal LC expressed TRANCE-R.

These data indicate that TRANCE, OPG and TRANCE-R are all present within epidermis at both protein and mRNA levels and lend support our hypothesis that this system may be critically involved in the regulation of epidermal LC survival. The distribution of TRANCE (produced by KC) and TRANCE-R (expressed on LC) suggests a cross-talk between these two cell types and implies a further key regulatory role for keratinocytes in the cutaneous immune response.

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Stem Cell Factor (SCF) Controls Mast Cell Accumulation At Sites Of Chronic Inflammation But Not Tumor Development

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Skin mast cells (MC) are known to accumulate at sites of chronic inflammation and tumor development. However, the mechanisms of MC hyperplasia in these processes is not yet fully understood. Stem cell factor (SCF) has been implicated to contribute to MC increases in various settings including chronic inflammation and tumor development via effects on its receptor kit expressed on MC. Here, we have used mice that do (Kit +/+) or do not express functional kit (KitW/KitW-v) to characterize the role of SCF in MC hyperplasia associated with chronic inflammation and tumor development. Normal mice were treated with TPA twice weekly, which resulted in pronounced inflammatory responses as assessed by quantitative histomorphometry and FACS analysis. Confirming previous reports we found that the induction of chronic inflammation is associated with markedly increased skin MC numbers, i.e. 5.5 $\pm$ 0.2 MC per microscopic field (MF) in untreated skin versus 33.9 $\pm$ 2.9 MC/MF in week 9 of treatment. Similar increases of MC numbers were found in the vicinity of developing skin tumors induced by topical DMBA/TPA treatment (6-fold increase at weeks 15 and 25). Interestingly, chronically inflamed skin in kit-deficient mice did not exhibit significantly increased MC numbers (0.03 $\pm$ 0.03 in week 0 and 0.2 $\pm$ 0.1 in week 9), indicating that SCF is required for MC hyperplasia at sites of chronic inflammation. Most notably, pronounced MC hyperplasia was found at sites of tumor development in kit-deficient mice, where numbers of MC were increased 125- and 260-fold in weeks 15 and 25, respectively. Our data show that SCF controls MC accumulation at sites of chronic inflammation but not tumor development. These findings suggest that skin tumor cells can induce MC hyperplasia by promoting MC recruitment, differentiation, and/or proliferation independent of SCF.

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Expression of the Stem Cell Markers Sca-1, CD34, CD45 and c-kit in Neonatal Mouse Skin

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Multipotent stem cells have been identified in multiple tissues including the skin. We have used FACS analysis to identify cells carrying stem cell markers in neonatal mice. Skin from neonatal FVB mice, 1–7 days of age, was digested with liberase and single cell suspensions prepared using the Medimachine (DakoCytomation). FACS analysis was carried out on fresh suspensions using antibodies specific for the early haemopoietic markers Sca-1, CD34, CD45 and c-kit. A major population of Sca-1$^+$ cells was identified, the percentage of which decreased with age from day 1 through day 7. In contrast, the percentage of c-kit$^+$ cells was constant over this time period. All Sca-1$^+$ cells were c-kit$^-$, MHC Class II$^-$ and consisted of two populations Sca-1$^+$CD34$^+$ and Sca-1$^+$CD34$^-$. About 2% of Sca-1$^+$ cells expressed the macrophage lineage markers CD45$^+$ CD11b$^+$ and were in the CD34 negative population. These results indicate CD45$^-$c-kit$^-$ cells expressing other stem cell markers in neonatal mouse skin are of non-haemopoietic origin.

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Melanoma Antigens Are Cross-Presented In Draining Lymph Nodes to CD8$^+$ T cells in the Absence of CD4$^+$ T Cell Activation : use of CD40-L to Correct the Defect

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Despite an apparent uncontrolled growth, host immune cells do not ignore tumors. However, interactions between tumor and immune cells remain poorly characterized. In the present work, we analyzed the spontaneous presentation of a model tumor antigen, ovalbumin (OVA), expressed in B16 melanoma cells. Upon adoptive transfer of OVA-specific T cells, OVA presentation in draining lymph nodes was observed in tumor-bearing mice. In this situation, a cytoplasmic form of OVA was more potently presented to CD8 + T cells than a membrane or a secreted form. This OVA presentation to T cells was dependent on host bone marrow-derived cells, indicating a cross-presentation mechanism. Importantly, this cross-presentation of B16-associated OVA to CD8 + T cells was achieved in the absence of presentation to CD4 + T cells. In this apparent absence of CD4 + T cell help, CD8 + T cells expanded but did not acquire effector functions (IFN and cytotoxicity). A systemic treatment with soluble CD40-L aimed at correcting the CD4 + T cell defect was shown to ameliorate the survival of B16-bearing mice. Altogether, this indicates that spontaneous immune response to tumors occurs but does not lead to the generation of cytotoxic effector T cells. This study indicates that treatment with immunostimulators such as CD40-L may have therapeutic values for melanoma.

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Early Recruitment of Cytotoxic CD8 + T Cell in Skin Lesion of Patients with Maculo-Papular Exanthema to Amoxicillin

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Cutaneous delayed-type hypersensitivity (DTH) drug reactions presenting as maculo-papular exanthema are due to activation in the skin of drug specific T lymphocytes. Penicillins, and especially amoxicillin (amox) are the most frequent drugs involved in these DTH reaction. However, the nature of effector T cells as well as the mechanisms by which they generate the skin inflammatory responses are not known. In this study, using immunohistochemistry and RT-PCR analysis, we followed the T cell recruitment and activation in the skin of positive patch test reactions to amox in 5 patients which have developed amox-induced maculo-papular exanthema. We show that CD8 + T cells are rapidly recruited in the skin at the site of drug application, before the onset of clinical signs of skin inflammation. This early CD8 + T cell recruitment is associated with: i) activation of effector T cells, assessed by transient IFN-γ mRNA expression and production of cytolytic proteins granzyme B and perforin; ii) apoptosis of keratinocytes demonstrated by Tunel staining. Collectively, these data suggest that drug-specific CD8 + T cells are effector cells in amox-induced DTH reactions in humans.

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Effects of Ageing on the Cutaneous Delayed Type Hypersensitivity Reaction to Tuberculin Purified Protein Derivative

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It is well recognised that ageing is associated with increased risk of infection and it is postulated that this occurs due to impaired cell-mediated immunity. The delayed type hypersensitivity (DTH) reaction to cutaneous injection of antigens is a widely used method to clinically assess cell-mediated immunity to infections such as tuberculosis. Previous studies have shown an increased percentage of negative DTH reactions to PPD and other antigens with age. We have previously demonstrated dissociation between the clinical DTH response (as measured by cutaneous erythema, induration and palpability) and cellular responses to tuberculin purified protein derivative (PPD) injection in the young. The aim of this study was therefore to establish whether the reduced clinical DTH response observed in the elderly is also associated with a reduced cellular response. One unit of PPD was injected in to the forearms of healthy individuals over the age of 70. Suction blisters were then induced over the site of injection at various time points. CD4 + T lymphocytes were isolated from the blister fluid and stained for Ki67, CD69 and IFN-γ after overnight stimulation with PPD. The cells were analysed using flow cytometry. Peripheral blood mononuclear cells (PBMCs) were collected prior to injection and stimulated with PPD overnight and for 5 days to assess for antigen specific cells (IFNγ staining) and cellular proliferation (thymidine incorporation) respectively. No individual in the elderly group had a clinical response to PPD injection. In particular, elderly individuals with good PBMC proliferative responses to PPD had low levels of expression of Ki67 and CD69 and no antigen specific cells in the blister fluid. These results were the same as those found in blister cells from elderly and young individuals with no PBMC proliferation to PPD. We have previously demonstrated that in young individuals the PBMC proliferative response to stimulation with PPD *in vitro* correlates well with the degree of cell proliferation and number of antigen specific cells at the site of PPD injection. The absence of clinical responses to PPD injection in the elderly is correlated with an absence of significant cellular proliferation and accumulation of PPD specific cells at the site of injection in spite of good PBMC responses to PPD. Thus, the DTH response may not accurately reflect cell-mediated responses to infection in the elderly and may represent other parameters such as changes in the local production of pro-inflammatory mediators, antigen presentation or cell migration to the skin.

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The Heat Shock Proteins 60 and 70 of *Propionibacterium acnes* are Antigens

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Propionibacterium acnes has been shown to be associated with the pathogenesis of inflammatory acne via different mechanisms. Its lymphocyte stimulating activity is mediated by mitogens and antigens. We have previously hypothesised that *P. acnes* heat shock proteins (HSPs) may initiate such responses. Purified HSP60 and HSP70 of *P. acnes* were used in a lymphocyte transformation assay time course experiment from day 3 to 7 with peripheral mononuclear cells (PBMNC) from patients with inflammatory acne and from cord blood (CBMNC) to investigate their T-cell stimulatory properties. A similar experiment was performed with and without pre-incubation with TU39, a monoclonal mouse anti-human MHC class II antibody, to block antigenic stimulation. 9/12 and 10/12 CBMNC were stimulated by HSP 60 and 70, respectively with 5/9 showing an early response. 3/9 samples showed two peaks of stimulation, suggesting two different mechanisms of lymphocyte activation. In contrast, only 3/11 of the PBMNC samples were stimulated by HSP 60 and 70 from day 5 onwards. Lymphocyte stimulation could be blocked completely by TU39. It can be concluded that *P. acnes* HSP 60 and 70 evoke an antigenic reaction and are not responsible for mitogenic activity. This is in accordance with immune reactions induced by mycobacterial HSP, which show a high degree of amino acid sequence homology with *P. acnes* HSP.

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Analysis of the Role for Type I Interferons in Psoriasis

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A common hypothesis states that the inflammatory skin lesions that characterise psoriasis patients are caused by aberrant signalling in genetically pre-disposed keratinocytes following triggers from cytokines or interactions with immune cells. Clinical case reports showed that psoriasis can be initiated or exacerbated in patients receiving IFN-α therapy. We recently demonstrated increased activation of the type I interferon (IFN-α/β) signalling pathway in lesional psoriasis epidermis. Stimulation of this pathway in normal and pre-disposed keratinocytes did not induce the inflammatory phenotype in keratinocytes. To study whether the pathological effects of IFN-α/β are mediated via cells of the immune system, we assayed the effects of IFN-α on skin antigen presenting cells (APC). APC that migrated from healthy skin in the presence of IFN-α showed a more mature phenotype, and were capable to induce T-cell proliferation in an allogeneic mixed leukocyte reaction. In addition, the effects of IFN-α on peripheral blood mononuclear cells (PBMC) were determined. In PBMC from healthy controls and psoriasis patients, IFN-α stimulation resulted in induction of a Th1-type cytokine profile with high levels of IFN-γ. Secretion of IL-10, a cytokine that plays an important role in the termination of (skin) inflammatory responses, was induced by IFN-α in healthy PBMC. In PBMC from psoriasis patients, however, IL-10 was not induced. Thus, since IFN-α/γ exerts many immunomodulatory effects, its pathological role in psoriasis is probably mediated via various cellular mechanisms, for example via an impaired induction of the anti-inflammatory IL-10.

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T Lymphocytes and NK Cells from Psoriasis Lesions Display a Distinct Chemokine Receptor Repertoire and Chemokine Responsiveness

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Psoriasis is a chronic inflammatory skin disease characterized by altered keratinocyte differentiation and prominent leukocyte infiltration. Type 1 cytokines released by both T and NK cells play a prominent role in disease expression. Immunohistochemical staining of acute psoriatic lesions revealed large numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells, as well as $CD56^+CD3^-$ NK cells in the papillary dermis and, to a lower extent, in the epidermis. A relevant percentage of $CD3^+$ T lymphocytes and NK cells were $CD25^+$ and $CD69^+$, indicating their activation state. Interestingly, the majority of $CD3^+$ T lymphocytes, but only on a minor fraction (15%) of NK cells isolated form psoriatic lesions expressed the CLA homing receptor. Chemokine receptors CXCR3 and CCR5 were highly expressed on skin infiltrating NK cells, which strongly migrated *in vitro* to CXCL10 and CCL5. In contrast, CCR4 expression and CCL17/CCL22 responsiveness was restricted to the $CD3^+$ T cell population. In addition, CCR6 levels and migratory capacity to CCL20 greatly prevailed in $CD3^+$ T lymphocytes compared to NK cells. No significant differences in the expression of CCR1, CCR2, CCR7 and CCR8, and *in vitro* chemotactic responses to the relevant chemokines were observed between the two cell types. Our data indicate that NK and T cells are actively recruited in psoriatic skin through distinct mechanisms. This finding can be relevant for the refinement of therapeutic approaches for chronic inflammatory skin diseases aimed to target leukocyte recruitment.

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Regulation of Th2 Cytokine Production by $CD4^+CD25^+$ T Cells Depends on Type and Concentration of Allergen

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Recently, we have shown that regulatory $CD4^+CD25^+$ T cells (Treg) are functional in most atopic patients with allergic rhinitis and are able to inhibit Th1 and Th2 cytokine production of $CD4^+CD25^-$ T cells. This study was set out to further analyze differences between atopic and nonatopic donors and allergen specificity, as well as the effect of allergen concentration and of the type of allergen. $CD4^+CD25^-$ T cells from healthy nonatopic controls or from grass pollen or wasp venom allergic donors were stimulated alone or in the presence of Treg with autologous mature monocyte-derived dendritic cells which were pulsed with different concentrations of the respective allergen. Treg from grass pollen allergic donors failed to inhibit proliferation but not cytokine production of $CD4^+CD25^-$ T cells at high antigen doses while Treg from nonatopic donors did not. The suppressed proliferation and cytokine production were almost antigen-unspecific. Proliferative responses and cytokine production of $CD4^+CD25^-$ T cells from wasp venom allergic patients were not inhibited at any concentration of wasp venom even after initiation of venom immunotherapy. The use of wasp venom- or phospholipase A_2-pulsed DC for stimulation of $CD4^+CD25^-$ T cells from donors which were not allergic to wasp stings did only result in an inhibited proliferation and Th2 cytokine production by Treg at ten fold lower than the optimal concentration compared to the use of grass pollen allergen-pulsed DC, while IFN-γ production was inhibited at all conditions. These data demonstrate that in allergic diseases the function of Treg is dependent on the concentration of the respective allergen with different thresholds for individual patients and allergens.

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Peripheral Blood Natural Killer Lymphocytes mediate both Natural Killer Cell Lysis and Antibody Dependent Cytotoxicity against Sezary Cells

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Although it has been shown that patients suffering from Sezary syndrome have a decreased natural killer (NK) lymphocyte activity, nothing has been reported concerning the sensitivity of Sezary cells to the granzyme B/perforin dependent cytotoxicity of NK cells. Since a decreased NK lysis can explain the severity of the disease, it is important to evaluate whether Sezary cells behave as potential targets for NK lymphocyte antitumoral activity and whether this function can be enhanced. Freshly isolated and IL-2/IL-12 activated peripheral blood purified NK cells were tested as effector lymphocytes against well-characterized Sezary tumoral cell lines in a chromium release cytotoxic assay. Further, these effector lymphocytes were studied for their ability to mediate antibody dependent cytotoxicity (ADCC), using the monoclonal antibody (mAb) IgG2a AZ158 that specifically recognizes KIR3DL2/CD158k expressed by Sezary cells. The results show that Sezary cells are susceptible to NK lysis with a maximum of specific lysis of 30%. However, we found that NK lymphocytes of some individuals were unable to kill Sezary cells, although these lymphocytes were functional. In such cases NK activity against Sezary cells could be induced with either IL-2, IL-12 or when incubating Sezary cells with an anti-MHC class I antibody F(ab)'2. Finally, we found that adding the AZ158 mAb enhances the lysis of these lymphoma tumoral cells. These results demonstrate for the first time that Sezary cells are susceptible to NK lysis. When the killing of malignant cells is lacking, it can be related to NK inhibitory receptors that recognize MHC class I antigens on the malignant cell. This inhibitory receptor effect is abolished when NK cells are stimulated with either IL-2 or IL-12. Finally, the mAb AZ158 can improve NK lysis of Sezary cells by ADCC. These findings raise the possibility to develop a novel therapeutic strategy in Sezary syndrome patients by stimulating their innate immunity.

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Pimecrolimus and Tacrolimus have Similar Potencies to Inhibit the Cytokine Production and Proliferation of Human T Cell Clones, Derived from Atopic Skin

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Pimecrolimus cream 1% and tacrolimus 0.1% and 0.03% ointment have been recently introduced into the market for the topical treatment of atopic eczema (AE) and efficacy of these two calcineurin inhibitors has been shown also in other T cell mediated inflammatory skin diseases. The objective of this study was to compare the *in vitro* potency of the two compounds to inhibit the proliferation and the synthesis of inflammatory cytokines by antigen-specific memory T cells, using cell clones derived from the skin of a patient suffering from AE. T cell proliferation, induced by dendritic cells loaded with specific antigen, was inhibited similarly by pimecrolimus and tacrolimus. The concentrations necessary to attain 50% inhibition (IC-50) differed by a factor of less than 2. In contrast, a ten- to twenty-fold higher IC-50 value was observed with cyclosporine A, used as a reference. A similar picture was obtained, when the effect of pimecrolimus and tacrolimus on cytokine synthesis (IL-2, IL-4, IL-5, IL-10, IFNγ and TNFα) was compared. The arithmetic means of the ratio IC-50 pimecrolimus / IC-50 tacrolimus were 1.53 ($\pm$ 0.26) or 1.66 ($\pm$ 0.36) when stimulating the T cells by anti-CD3 monoclonal antibody plus phorbol ester or by antigen-presenting dendritic cells, respectively. In conclusion, the intrinsic *in vitro* potency of pimecrolimus and tacrolimus to inhibit T cell activation, a key step in the pathomechanism of AE, is very similar.

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Budesonide, but not Tacrolimus, Affects the Immune Functions of Normal Human Keratinocytes

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Immunosuppressant therapy is widely used in the treatment of inflammatory skin diseases such as psoriasis and atopic dermatitis. Besides its therapeutic effects, application of topical anti-inflammatory drugs may render the epidermis more vulnerable to invading pathogens by suppressing innate immune responses in keratinocytes such as cytokine production or Toll-like receptor (TLR) expression of cells.

In order to evaluate and compare the immunosuppressive effects of different immunosuppressant drugs on keratinocytes we treated lipopolysaccharide (LPS) stimulated and unstimulated normal human keratinocytes with the synthetic corticosteroid budesonide and the macrolide lactone tacrolimus. The expressions of the pattern recognition receptors (PRRs) TLR2 and TLR4 were measured by quantitative RT-PCR and pro-inflammatory cytokines IL-1α, IL-8 and TNF-α were monitored by quantitative RT-PCR and by ELISA.

In unstimulated normal human keratinocytes IL-8 and TNF-α mRNA expressions were suppressed, TLR2 mRNA expression was induced by budesonide. Tacrolimus showed no effect. In LPS induced keratinocytes budesonide slightly hindered the increase of IL-8 mRNA expression and further increased (4–6 fold) the amount of TLR2 mRNA elevated by LPS. There were no changes in TLR4 mRNA expressions. Suppressive effect of budesonide on IL-8 gene expression was also revealed at protein level in LPS treated human keratinocytes.

Although tacrolimus and budesonide are both effective treatments in inflammatory skin diseases, these data provide explanation for the difference observed in local adverse effects of these two topical immunosuppressants.

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T-cell infiltration and Epidermal Proliferation In Lesional Plaque Psoriasis During Topical Treatment With Clobetasol-17 Propionate Ointment

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In psoriasis the appearance of T-cell subsets and epidermal hyperproliferation are key phenomena. It is known that CD8+, CD45RO+ and CD2+ T-cells appear in an early phase of the evolving psoriatic lesion. The dynamics of reduction of T-cell subsets in relation to the decrease of epidermal proliferation in response to treatments are less well understood.

Therefore, our aim was to investigate the dynamics of changes in T-cell subsets in relation to epidermal proliferation and clinical severity in lesional chronic plaque psoriasis during topical treatment with the ultra-potent corticosteroid clobetasol-17 propionate ointment.

Seven psoriasis patients were treated for 14 days with clobetasol-17 propionate ointment, twice daily. Four punch biopsies were taken at baseline and 3, 7 and 14 days after the start of the therapy. PASI- and SUM-scores were calculated. Epidermal proliferation marker Ki-67 and CD4+, CD8+, CD45RO+, CD2+ T-cells were quantified using immunohistochemical techniques and compared with baseline values using image analysis.

The results show a rapid reduction of PASI and SUM-score, reaching a 54% and 60% reduction after 2 weeks treatment, respectively ($p<0{,}01$). Already after 3 days treatment a 47% reduction of Ki-67 positive nuclei was observed ($p<0{,}01$). Only after 14 days, in the epidermis, all investigated T-cell subsets were reduced significantly ($p<0{,}05$). In the dermis, however, treatment had resulted in a significant decrease of CD4+, CD45RO+ and CD2+ T-cells, with a selective persistence of CD8+ T-cells.

We conclude that in lesional psoriasis, reduction of clinical severity and epidermal proliferation in the early phase of topical corticosteroid therapy cannot primarily be the result of decreased subsets of T-cells. Furthermore, selective persistence of dermal CD8+ T-cells after corticosteroid treatment was observed, which might account for relatively fast relapses after cessation of treatment.

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Enhanced Expression of Skin Homing Molecules CLA and CCR4 on CD8+ T Cells in Cutaneous Lupus Erythematosus

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Introduction. Skin infiltrating T lymphocytes are supposed to play a major role in the pathogenesis of cutaneous lupus erythematosus (LE), but little is known about their distinct character and their skin homing properties.

Methods. The expression of the cutaneous lymphocyte antigen (CLA) and the chemokine receptor 4 (CCR4) on lesional and circulating T cells in cutaneous LE patients was analyzed by immunohistochemistry and flowcytometry.

Results. We found a strong expression of CLA and CCR4 in the skin of patients with chronic discoid LE (CDLE) (CLA: 271 cells per high power magnification $\pm$ 32.3 in CDLE to 20 $\pm$ 5 cells per HPM in healthy controls, $p<0.01$; CCR4: 52 $\pm$ 9 to 2.6 $\pm$ 0.5, $p<0.01$). Histomorphologically, cytotoxic CD8+ T cells, largely expressing granzyme B, invaded basal layers of the epidermis. CCR4 and CLA coexpression on circulating CD8 T cells was upregulated specifically in disseminated CDLE (19.6% $\pm$ 0.6 in dCDLE to 1.5% $\pm$ 0.5 in healthy controls, $p<0.01$).

Discussion. We assume that the expression of CCR4 and CLA on CD8+ T cells plays an important role in the pathogenesis of cutaneous LE. Our results suggest a role for CD8+ CLA+ CCR4+ T cells in specific cytotoxic destruction of epidermal structures in scarring CDLE.

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The Maturation And Migration Capacity Of Antigen Presenting Cells Of The Skin Remains Unchanged After Treatment With Pimecrolimus

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Migration of antigen-laden Langerhans cells (LC) into draining lymph nodes is a pivotal step in the development of the primary immune response in contact hypersensitivity (CHS). We have examined the effects of the calcineurin inhibitor pimecrolimus on the migration and maturation capacity of LC *in vitro* and *in vivo*. Ears of BALB/c mice were treated topically with 10 µl pimecrolimus 1% or with vehicle, twice at an interval of 8 h on day 1. Forty hours later, the ears were dissected and the dorsal, mechanically separated, sides floated on culture medium for 3 days prior to analysis. LC in epidermal/dermal sheets and migrated LC and dermal dendritic cells (DDC) in the culture medium of pimecrolimus- and vehicle-treated samples were similar, both in numbers and degree of maturation. Antigen-induced migration of LC into the draining lymph nodes was studied in 12 mice each treated orally with 90 mg/kg pimecrolimus or vehicle 2 h before and 4 h after FITC application. After 24 h, the proportion of antigen-laden $FITC^+MHC\ II^+$ cells in lymph nodes were not statistically different in pimecrolimus- and vehicle-treated mice (1.40 vs. 1.54%). In 12 mice treated intraperitoneally with 200 µg anti-LFA-1 antibody (M 17.4), used as a positive control, only 0.25% $FITC^+MHC\ II^+$ cells were detected in lymph nodes ($p>0.05$). Thus neither topically nor systemically administered pimecrolimus interferes with the migratory and maturation capacity of LC/DDC. This is in line with previous data, which showed that oral treatment of mice with doses up to 120 mg/kg pimecrolimus did not impair the induction phase of CH in contrast to cyclosporine A and tacrolimus, whereas the elicitation phase was potently inhibited with lower doses. These data imply that pimecrolimus does not impair cutaneous immunosurveillance.

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Distinct Expression of Adhesion Molecules on Monocytes of Patients With Inflammatory Skin Diseases

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Monocytes are considered as direct precursor cells of inflammatory dendritic cell subtypes, which are recruited from the blood into the skin in inflammatory skin diseases such as atopic dermatitis and psoriasis. It is assumed that chemotactic signals initiate a multistep cascade via different adhesion receptors which consists of "tethering" and "rolling" mediated by selectins, α-integrins and β-integrins. The "arrest" of the cells is in main part dependent on the activation of integrins. Furthermore it is hypothesised that cell activation and signalling involves tetraspanins.

Since different activation signals play a pivotal role in the recruitment phase of allergic-inflammatory skin diseases and these initial steps are of major interest for the development of preventive therapeutic strategies we started to analyse the expression of adhesion molecules on monocytes of patients at the exacerbation state of atopic dermatitis ($n=10$) or psoriasis ($n=9$) in comparison to healthy, non-atopic control donors ($n=7$). With the help of detailed phenotypical analyses, differences in the surface expression of α-integrins, β-integrins, selectins, tetraspanins, Fc receptors, costimulatory molecules and major histocompatibility class I and II molecules have been evaluated.

An enhanced expression of the α-integrin CD49a, CD49b and CD49c and the β-integrin CD41 and CD61 has been detected in both, monocytes from patients with atopic dermatitis or psoriasis and is therefore considered as a common sign for inflammation. Characteristic for monocytes of patients with atopic dermatitis was an increased expression of the tetraspanin superfamily CD9, CD37, CD81 and CD151. In contrast, monocytes of patients with psoriasis displayed a high surface expression of the selectin CD62L and CD62P and the thrombospondin receptor CD36.

From these data we conclude, that (I) an increased expression of integrins on peripheral blood monocytes of patients with inflammatory skin diseases might promote enhanced skin homing of these cells, (II) high expression of tetraspanins observable on monocytes of patients with atopic dermatitis might favour a rapid transendothelial migration of these cells while (III) high levels of L- and P-selectin on the surface of monocytes of patients with psoriasis might form the basis for a rapid recruitment process in which selectin-dependent leucocyte rolling plays a major role.

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Therapeutic Efficiency of the Infusion of TIL Specific for Melanoma Antigens

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Adoptive therapy of cancer has been tested mostly in advanced cancer patients using tumor-infiltrating lymphocytes (TIL). Limited clinical success has been discouraging, although the poor TIL specificity and/or the high tumor burden might explain it. We recently show the efficiency of tumor reactive TIL infusion to prevent further development of the melanoma disease in stage III melanoma patients, after complete tumor resection. Here we analyze the survival without relapse of these same patients according to TIL specificity and lymph node invasion. In this study, we show that survival without relapse correlates not only with tumor burden but also with the presence of melanoma specific lymphocytes among infused TIL. We also characterized the antigenic specificities recognized by these TIL in order to determine which are the most interesting melanoma antigens to target in immunotherapy protocols.

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15-lipoxygenase is Expressed in the Skin of Patients with Atopic Dermatitis and Contact Dermatitis

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Background. The enzyme 15-liopxygenase (15-LO) catalyzes the conversion of arachidonic acid to 15-HPETE and other related metabolites. The 15-LO has been shown to exist in reticulocytes, dendritic cells, eosinophils, and also airway epithelial cells in asthma, where it is believed to have a pro-inflammatory effect. Since the 15-LO is activated in asthma, and since the metabolism of arachidonic acid is changed in the inflammatory disease psoriasis, it would be of interesting to investigate 15-LO also in allergic skin disorders.

Objective. 1) to investigate the presence of 15-LO in the skin of patients with atopic dermatitis (AD), contact dermatitis (CD), psoriasis and healthy individuals, 2) to explore whether 15-LO is up regulated when the skin is stimulated with the allergens and 3) to find out which cells can express 15-LO in the skin.

Material and methods. Biopsies were taken from normal and atopy patch tested (Malassezia) skin from 15 patients with AD and 8 healthy controls, lesional skin from 11 AD patients, normal and patch tested skin (Ni) from 5 patients with CD and normal and lesional skin from 5 patients with psoriasis.

Results. Few dermal cells expressed 15-LO in biopsies from normal skin of patients and healthy controls. Positive epidermal cells were rarely seen in healthy skin. Even though an increase in the number of dermal and epidermal 15-LO+ cells was seen after atopy patch test in both patients with AD and healthy individuals, the number of 15-LO+ cells in the AD group was significantly higher than in the control group. The number of 15-LO+ in lesional AD skin was significantly higher than in non-lesional skin. Patients with CD showed a high number of dermal and epidermal cells with strong expression of 15-LO after patch test with Ni. In biopsies from lesional psoriasis skin only few cells were positive, mostly showing a week 15-LO-expression. The number of 15-LO+ was sometimes lower than in non-lesional skin from the same patient. Double stained biopsies taken from the eczema lesions and patch tested skin from patients with AD and CD revealed that 15-LO was expressed by Cd1a+ dendritic cells, eosinophils and a minority of the macrophages. Tryptase+ mast cells and T cells were 15-LO-.

Conclusion. Stimulation with an allergen induced 15-LO expression not only in patients with allergic eczema, but also in healthy individuals. Nevertheless, the 15-LO expression was higher in both AD and CD than in the skin of healthy controls. Since 15-LO is produced by dendritic cells and eosinophils, cell types known to be present in allergic eczema, it might play a role in inducing allergic skin inflammation.

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Identification and Characterization of ''pDC-like Cells'' in Normal Mouse Skin and Melanomas Treated with Imiquimod

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Dendritic cells (DC) constitute a heterogeneous population of antigen-presenting cells (APCs) found virtually in every tissue and organ. They link innate and adaptive immunity by inducing appropriate immune responses upon the recognition of invading pathogens, thus acting as "nature's adjuvant". Among the different subsets of DCs described in humans and mice, epidermal Langerhans cells and dermal DCs represent the only DC populations resident in normal skin.

Here, we describe a population of $CD4^+/CD3^-$ "plasmacytoid DC (pDC)-like cells" which accumulate in the dermis and spleens of mice topically treated with Imiquimod, a low molecular weight immune response modifier with potent anti-viral and anti-tumor activities. These $CD4^+/CD3^-$ cells co-express GR-1, B220, MHC-II and, to a lesser extent, CD11c and display phenotypic features of pDCs described in lymphoid organs. The accumulation of pDC-like cells after Imiquimod treatment was not only detected in normal skin, but also in intradermally-induced melanomas. Imiquimod treatment leads either to complete regression or to a significant reduction of the tumors. The number of pDCs correlates well with the clinical response of the tumors to the drug suggesting that the anti-tumor effects of Imiquimod could at least partly be mediated by the recruitment of pDC-like cells to the skin. Therefore, strategies aimed at activating and directing these cells into neoplastic tissues may be a promising and novel approach for the immunotherapy of various types of cancer.

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Pimecrolimus Ameliorates Disease in a Prophylactic Mouse Model of Lupus

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Systemic lupus erythematosus is a multisystem autoimmune disease with a high unmet medical need that effects many organs including kidney and skin. Since oral pimecrolimus (PIM) was found to be safe and effective for treatment of atopic dermatitis and psoriasis in man and in animal models of arthritis and inflammatory bowel disease, we have investigated its effect in the MRL/MpJ-*Tnfrsf6*^{lpr} (MRL/lpr) model of SLE. We have compared the effects of PIM with those of cyclophosphamide (CP), a drug that is used effectively for the treatment of human lupus nephritis but that has severe side effects. Data from 2 independent studies where mice were given 30 (PIM 30) or 50 (PIM 50) mg/kg/d PIM orally during an 8 week period when disease symptoms develop suggest that both PIM and CP are equally effective in reducing disease progression and severity. Proteinuria scores were significantly reduced at PIM 50 ($p<0.01$) and PIM 30 ($p<0.05$). Glomerulopathology severity scores were similarly reduced: PIM 50 ($p<0.001$) and PIM 30 ($p<0.05$). In MRL/*lpr* treated mice both CP and PIM 50 significantly decreased ($p<0.001$) the lymph node population of CD4-CD8-CD3+, double negative T cells which is an abnormal T cell subset that accumulates over time due to the *lpr* mutation. The reduction in the generalized hypergammaglobulinemia characteristic of MRL/*lpr* mice which was greatly reduced by CP compared to placebo controls ($p<0.001$) was seen as a trend with PIM 50 as was the production of anti-ds DNA antibodies. A trend to lower levels of Rheumatoid Factor was evident in the PIM 50 and CP groups versus placebo treated controls. This study demonstrates that testing in the early stages of disease in the MRL/*lpr* lupus mouse is a suitable and relatively fast prophylactic model for drug profiling. Pimecrolimus improves key pathological parameters of lupus in this model which suggests its therapeutic potential for lupus in man.

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CD4+ CD56+ Blastic Tumor Cells Express CD101/BC27 Antigen

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CD4+CD56+ tumors are rare hematopoietic malignancies characterized by a frequent skin and blood involvement and a rapid aggressive course with bone marrow infiltration. By contrast with myeloid/NK cell acute leukemias, CD4+CD56+ malignancies usually lack the expression of conventional myeloid and lymphoid T- and B-cell markers. Several recent studies reported phenotypic and functional similarities between these CD4+CD56+ leukemic cells and a subtype of dendritic cells (DC), so-called plasmacytoid dendritic cells or type 2 dendritic cells (DC2). CD101 is a 200kDa disulfide linked homodimer. In normal individuals, it is expressed by a minor subset of circulating T-lymphocytes, by intestinal mucosal T-lymphocytes and by a major DC cells subset including DC2 cells. CD101 antigen plays a major role in the activation of T-lymphocytes by DC and inhibits T-cell proliferation via IL-10 secretion. In this study we analysed, using immunochemistry on cryostat tumor sections, the CD101 expression in the cutaneous tumor infiltrate of 17 patients with CD4+CD56+ malignancy. In addition, we performed three colour flow cytometry analysis on the peripheral blood mononuclear cells (PBMC) in one of these patients. We found CD101-stained cells with blastic morphology in 13 of 17 (76%) tumor cutaneous infiltrates. 90% of PBMC, corresponding to circulating tumor cells, co-expressed CD101, CD4 and CD56 antigens in the analysed blood sample. In conclusion, we showed, for the first time that CD4+CD56+ tumor cells express the CD101 marker. This expression is one more evidence of the identity between DC2 cells and CD4+CD56+ tumor cells. Moreover, CD101 could represent a useful tool for the diagnosis of the CD4+CD56+ blastic tumors. The next step will be to determine the role of CD101 in the pathogenesis of this tumor. It can be speculated that CD101 could promote the progression of the disease by inducing a tumor-specific tolerance.

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Exposure To UVB Radiation Induces An Alteration In Blood Dendritic Cell Subtypes

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Exposure to ultraviolet radiation (UVR) causes alterations of cutaneous and systemic immunity. The aim of our study was to assess the influence of UVB radiation on the phenotypes of blood dendritic cells (BDCs).

Healthy volunteers (30) were irradiated (whole body) with a dose of 0.7 MED (minimal erythemal dose) of UVB for 10 consecutive days. 24 hours after a final exposure, local irradiation (3 MED) on buttock skin was performed. Blood samples were taken before the first exposure and 24 hours after final exposure of 0.7 MED and 24 hours after 3 MED irradiation. The three main subsets of BDCs were distinguished by flow cytometry: BDCA-2+/CD123+/HLA-DR+ (plasmacytoid, PDCs), and two myeloid subtypes: BDCA-1+/CD1c+/HLA-DR+ (MDC1s) and BDCA3+/CD86+/HLA-DR+ (MDC2s). The percentages and absolute numbers of DCs and their subsets were calculated.

The median percentage of particular DC subsets before irradiation was as following: MDC1s- 0.34%, MDC2s –0.1%, PDCs-0.57% of PBMC. Both after 10 days of 0.7 MED and after a single dose of 3 MED a slight increase in MDC1 subtype was noted (median-0.39 and median-0.4, respectively), however not statistically significant. A statistically significant drop of percentage of MDC2s was found after 10 days of irradiation (median-0.08) and after 3 MED (median-0.05). PDC rate was also decreased both after 10 days and 3 MED irradiation (median-0.52 and median-0.47; respectively), but the differences were not statistically significant.

Conclusion. Exposure to UVB induced a drop in the percentage of BDCs in healthy human individuals, especially apparent in the MDC2 subtype.

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Expression of Sensory Neuropeptides CGRP, Substance P and NK-1 Receptor in Human Contact Dermatitis

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There is increasing evidence of an interaction between the nervous and the immune systems. Sensory neuropeptides (CGRP, calcitonin gene-related peptide, and substance P) have been shown to modulate allergic contact dermatitis in mice. The aim of this study was to investigate the expression of CGRP, substance P and its NK-1 (neurokinin-1) R (receptor) in human contact dermatitis by using immunohistochemistry and a streptavidin-biotin method. Biopsies from positive epicutaneous patch test reactions to nickel sulphate ($n=10$) and from control skin ($n=9$) in nickel-allergic patients, were fixed in 10% formalin with 0.2% picric acid and processed for immunohistochemistry.

The number of CGRP immunoreactive nerve fibers in the papillary dermis showed a tendency to a decrease in the eczematous (5.5 ± 2.9 fibers/section) compared to control (7.1 ± 4.0) skin, while there was no difference between the number of substance P positive fibers in the inflamed (4.2 ± 1.7) compared to control skin (4.4 ± 2.1). Semiquantification of substance P and NK-1R positive papillary dermal mononuclear cells showed an increase in the eczematous (+++) skin in contrast to control (+) skin. In addition, NK-1R immunoreactivity was higher in basal keratinocytes in inflamed compared to control skin. The intensity of this immunoreactivity appeared to be increased with more intense inflammation.

Our results indicate that CGRP and substance P participate in the pathophysiology of human allergic contact dermatitis.

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Cyclophosphamide(Cy) Accelerates Atopic Dermatitis(AD) Like Eruptions in DS-Nh Mice

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Background. DS-Nh mice raised under conventional condition spontaneously develop dermatitis similar to human atopic dermatitis(AD), which is thought to be associated with staphylococcal infection. In our previous study, we demonstrated DS-Nh mice, which had received the treatment of TNCB, developed more severe AD-like skin eruptions earlier as compared with the controls. Skin eruptions were observed not only on the area painted with TNCB, but also on the face and neck areas on which were not painted, and the serum levels of total IgE elevated in correlation with the severity of the clinical skin conditions.

Objective. To examine effect of Cyclophosphamide(Cy) treatment on AD-like eruptions of DS-Nh mice, which were induced by TNCB sensitization.

Method. We applied Cy (200 mg/kg) intraperitoneally 2 days before TNCB sensitization. Then, low dose of TNCB were applied epicutaneously once a week. We examined skin eruption score, serum total IgE levels, and cytokine production from splenocytes and lymph nodes cells cultured with SEB or ConA by ELISA. We also conducted Cy effect on the T cells by FACS analysis.

Results & Conclusion. Mice treated with Cy developed severe AD-like skin eruptions in comparison with non-treated ones. The levels of INF-gamma and IL-13 were elevated in the Cy treated mice, although serum levels of total IgE were comparable between two groups. Total cell number of Cy treated spleen and lymph nodes were decreased at day 3, and proportion of CD25+CD4+ T cells in total CD4+ T cells were also significantly decreased.

In this experiment, we revealed that treatment of Cy accelerates the skin symptoms (AD-like skin eruptions) induced by low dose of TNCB application. Moreover, the AD-like skin eruptions continued more than 3 weeks after stopped applying TNCB, and the eruptions were seen another lesion of TNCB applied area. Examined cytokine production, IFN-gamma that is the Th1 type cytokine elevated rather than IL-4 (Th2 type cytokine). The amounts of IFN-gamma and IL-13 production by splenocytes or lymph nodes cells from Cy treated mice were significantly larger than those of control mice. It is presumed that Th1-predominant mechanisms and CD25+CD4+ T regulatory cells might be involved in the development of AD-like eruptions rather than IgE production.

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Lichen Sclerosus: Detection of Activated Cytotoxic Cells in the Cellular Infiltrates

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Granzyme B and perforin messenger RNA (mRNA) expression has been shown to be a specific *in vivo* activation marker for cytotoxic cells. The aim of this study was to assess the contribution of cell-mediated cytotoxicity in the pathogenesis of lichen sclerosus. *In situ* hybridisation and immunohistochemistry were performed on serial tissue sections of lesional skin biopsies and normal skin as a control. Immunhistochemical staining showed that the cellular infiltrate of diseased skin consisted predominantenly of T cells (CD3+) and some B cells (CD20+). Among T cells CD4+ cells were more abundant than CD8+ cells. In normal skin samples perforin and granzyme B mRNA expressing cells were only rarely found. In contrast, in skin biopsies from diseased tissue specimens a high percentage of infiltrating T cells expressed mRNA for perforin and granzyme B. The perforin and granzyme B expressing cells were found in the dermal infiltrate and intraepidermally in close proximity to keratinocytes suggesting *in situ* activation of these cells. These findings provide evidence that cell mediated cytotoxicity plays a significant role in tissue destruction in lichen sclerosus.

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Mature and Immature Langerhans Cells are Reduced in Vitiligo

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In vitiligo patients melanocyte-specific autoantibodies as well as cytotoxic T cells have been found in the peripheral blood. The role of professional antigen-presenting cells, eg. Langerhans cells (LCs), in the pathogenesis of vitiligo is still unknown. We investigated in the present study subpopulations of LCs in vitiligo. Using immunohistochemistry we examined CD1a and Langerin expression in 6 healthy controls and in lesional and non-lesional skin of 10 patients with vitiligo. The number of CD1a+ or Langerin+ LCs in the epidermis of lesional skin was significantly reduced compared with non-lesional or control skin. Furthermore an increased Langerin+/CD1a+ ratio was observed in the epidermis. No difference in the number of CD1a+ or Langerin+ cells of the dermis was noted between the different groups. In lesional and non-lesional skin of vitiligo patients CD1a+ and Langerin+ cells were often located in more basal layers of the epidermis than in control skin.

Our data demonstrate a reduction of mature, Langerin+ as well as immature, CD1a+ LCs in lesional skin of vitiligo patients. This may either be explained by cytotoxicity or by decreased immigration respectively increased emigration of LCs.

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Phenotypical Study of Lymphocytes Infiltrating the Skin in Atopic Dermatitis after Expansion *ex vivo*

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Background. Atopic dermatitis (AD) is a frequent chronic inflammatory dermatological condition. AD skin lesions are characterized by a T-cell infiltrate. However, AD pathogenesis is not yet totally understood. Few studies have analysed the phenotypic characteristics of the lymphocytes infiltrating the skin in AD, and most have been performed after cell expansion *ex vivo*.

Objective. To investigate the phenotype of these cells, and to evaluate if the *ex vivo* culture conditions induce the growth of selective lymphocyte subsets.

Methods. T lymphocytes were expanded from skin biopsies of AD (12 patients), non-AD eczema (7 patients) and normal skin (2 patients), when cultured in RPMI 1640 medium with 10% human AB serum, antibiotics, fungicide and IL-2, initially associated to IL-4. In addition, peripheral blood lymphocytes (PBL) from a healthy donor were divided in three groups and cultured during 5 days under these same conditions unless for cytokines: IL-2 alone (group 1), IL-2 and IL-4 (group 2), IL-4 alone (group 3). The phenotypic analysis was done by flow cytometry using fluorescent monoclonal antibodies.

Results. We observed no difference between the phenotype of lymphocytes infiltrating the skin in AD, non-AD eczema and normal skin. In most cases, there was a clear predominance of CD4 over CD8 T-cells. Double positive CD4+ and CD8+ T cells could be detected in low proportions only for some patients (3 AD, 6 non-AD eczema, 1 normal skin), as opposite to what has previously been reported. All the tested patients presented double positive CD4+ and Granzyme B+ cells in significant proportions.

The three groups of differently cultured PBL presented different phenotypes: group 1 showed an increased proportion of double positive CD4+ and Granzyme B+ (58,4%) compared with the same cells before culture or with group 2 (16%) or group 3 (13%).

Conclusions. Culture conditions which enable the expansion of skin-infiltrating lymphocytes can induce the growth of selective lymphocyte subset and the expression of cytotoxic markers among CD4+ T-cells. Thus, only *in situ* phenotypic analysis are appropriate for studying the phenotype of the skin infiltrating lymphocytes, in order to understand the pathogenesis of the disease.

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***In situ* Expression Of LAT (Linker For Activation Of T-Cells) In Pathological Human Skin With T-Lymphoid Infiltrate**

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LAT is a 36-kD transmembrane protein that plays an important role in linking engagement of the TCR to the biochemical events of T cell activation. It has been shown that LAT reacts with human T cells in normal and neoplastic lymphoid tissues, without restriction to any T cell subpopulation. These findings have suggested that the expression of LAT *in vivo* represents a valuable addition to the panel of immunohistochemical markers that can be used for immunostaining T-cells. The expression of LAT has not yet been studied in human pathological skin conditions. In this study we present our experience concerning LAT expression in both neoplastic and inflammatory dermatoses using an immunohistochemical approach on frozen sections from 50 patients. In our study, a variable reduction in LAT expression was observed in almost all the inflammatory and neoplastic skin conditions investigated, irrespective of the particular disease. Our study indicates that LAT-negative T cells are more common within the skin T-lymphoid infiltrate than was previously demonstrated in both normal and neoplastic lymphoid tissues. These findings suggest that, using a conventional immonoenzymatic approach on fresh frozen sections, LAT staining is an unreliable marker for the identification of T cells in human pathological skin conditions.

199 [Oral 018]

CXCR6 and its Ligand CXCL16 Mediate T Cell Recruitment into Psoriatic Skin

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In recent years, much effort in research has focussed on the identification of tissue and disease specific chemokine receptors. Targeting major receptors of an inflammatory process will offer for the first time the possibility for disease specific therapeutic interventions. To this end, chemokine receptor expression was analyzed in psoriatic skin derived T cell lines and compared to PBL derived lines from the same donors. Remarkably, CXCR6 was expressed more prominently in the skin derived T cell lines, both in the CD4 and CD8 subset. Moreover, CXCR6 expression was exclusively found in the $CCR7^-$ population, marking them as effector memory T cells ready to respond vigorously to activation. Indeed, these CXCR6 T cell lines produced Th1 cytokines like IL-2 and IFN-γ but no Th2 cytokines like IL-4. In the contrary, Th cells from atopic dermatitis with a Th2 cytokine pattern were all CXCR6 negative. The $CXCR6^+$ $CD4^+$ and $CD8^+$ T cells from psoriatic skin responded to the CXCR6 ligand CXCL16 as determined by intracellular Calcium mobilization assays. Moreover, psoriatic skin derived T cells migrated to CXCL16 *in vitro* and, *in vivo*, CXCL16 recruited human T cells to human skin grafts previously transplanted onto SCID mice. Analysis of expression profiles by immunohistochemistry demonstrated the presence $CXCR6^+$ T cells in psoriatic skin. Comparing different leukocyte subsets as well as resident skin cells for the production of CXCL16, monocytes were identified as the major source of CXCL16 by intracellular FACS staining and ELISA. Consequently, $CXCR6^+$ T cells migrated in response to monocyte supernatants. As monocytes may produce several T cell attracting chemokines, the relevance of this finding was further investigated by neutralizing CXCL16. Indeed, addition of blocking anti-CXCL16 mAbs to the supernatants or CXCR6 desensitization by adding an excess of CXCL16 to the T cells strongly inhibited this migration. These investigations indicate that CXCL16 and CXCR6 are new and apparently important players in the process of psoriatic inflammation. Thus, targeting CXCR6 in psoriasis may be a novel therapeutic approach which not only reduces skin inflammation but is also devoid of systemic side effects as seen with immunosuppressants.

200 [Oral 017]

CCL18 is Selectively Expressed in Atopic Dermatitis and Mediates Skin Homing of Human Memory T Cells

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CCL18 is a homeostatic chemokine produced by human monocytes and dendritic cells (DC) in lymphoid tissues. The expression of CCL18 is induced by IL-4, which is a cytokine characteristically present in allergic reactions. Atopic dermatitis (AD) is an inflammatory skin disease, which is characterized by lesional infiltrates of IL-4 producing Th cells and therefore AD represents a suitable environment for CCL18 upregulation.

We found CCL18 expression in skin biopsies of AD patients but not in normal or psoriatic skin. CCL18 was specifically expressed by monocytes and dendritic cells in the dermis of AD skin and by Langerhans cells and inflammatory dendritic epidermal cells in the epidermis. Furthermore, serum levels of CCL18 were significantly increased in AD patients compared to healthy controls and a higher percentage of monocytes and dendritic cells secreted CCL18 in IL-4 stimulated PBMC of atopic donors compared to healthy individuals. Interestingly, the higher CCL18 serum levels were observed in moderate compared to very severe disease stages, suggesting that CCL18 could be used as a biomarker for AD. In addition, fluorescently labeled CCL18 bound not only to naïve T cells in peripheral blood but also to memory Th cells isolated from the skin of AD patients and induced the migration of these memory Th cells *in vitro* and in human skin transplanted onto SCID-mice. These findings reveal a novel inflammatory role of this chemokine mediating skin-homing of a subpopulation of human memory T cells.

201 [Oral 021]

Treatment with the Angiogenesis Inhibitor Vasostatin Resulted in Decreased Delayed-Type Hypersensitivity Reactions in the Ear Skin of Mice

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Angiogenesis and increased vascular leakage are characteristic features in the context of inflammation. The purpose of our study was to examine the effects of an antiangiogenic treatment on inflammatory processes *in vitro* and *in vivo*. Vasostatin, an endothelial cell inhibitory factor present in culture supernatants of Epstein-Barr-virus-immortalized cells was recently identified as the amino terminal domain of Calreticulin (amino acids 1-180) and was found to inhibit proliferation of endothelial cells and to suppress neovascularization and tumor growth *in vivo*. We induced delayed-type hypersensitivity (DTH) reactions in the ear skin of wildtype FVB mice with and without vasostatin treatment in order to study the extent of vascularity, vascular leakage and leukocyte-recruitment. Twenty-four hours after the initial challenge, the vasostatin-treated mice exhibited 38% less edema when compared to the untreated mice. This trend was observed throughout the investigated recovery period of 7 days. In an Evans blue leakage assay we observed 43% less plasma leakage. Furthermore, we found a significant decrease in blood vessel density in the inflamed ears of the vasostatin-treated mice. Vessel spread analysis combined with three-dimensional vessel imaging using whole mounts of ears following lectin perfusions demonstrated fewer larger vessels in the treated group compared to the control animals. The observed decreased number of adherent leukocytes in the lectin perfused ears of treated animals is in accordance with intravital microcopy studies showing a significant decrease in the rolling fraction of leukocytes in vasostatin-treated mice. Electron microscopy studies indicated that Vasostatin may prevent leakage by maintaining the integrity of interendothelial junctions. Furthermore we investigated the effect of Vasostatin on macromolecular permeability of human dermal microvascular endothelial cell (HDMEC) monolayers stimulated with the permeability-inducing agent VEGF-A. Vasostatin potently inhibited VEGF-induced permeability implying that the mechanism responsible for the observed *in vivo* results may involve direct activity of vasostatin on the endothelial cells. Our study suggests that therapeutic approaches using angiogenesis inhibitors could prove beneficial in the treatment of cutaneous inflammation.

202 [Oral 039]

Thrombospondin 1 Modulates Inflammation, Vascular Leakage and Vascular Remodeling During Experimental Delayed-Type Hypersensitivity Reactions

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Thrombospondin 1 (TSP-1) is a potent endogenous angiogenesis inhibitor that is thought to play an important role in maintaining cutaneous vascular quiescence. However, TSP-1 has been detected in inflammatory diseases such as arthritis and has been described as pro-angiogenic and pro-inflammatory in some experimental models. To elucidate the function of TSP-1 in a cutaneous inflammation, we studied the expression of TSP-1 in human allergic contact dermatitis (ACD) induced by patch testing. 72 hours after the onset of the inflammation, both TSP-1 protein and mRNA expression were potently upregulated in the inflamed lesions when compared to normal skin taken from the same patient. In-situ hybridization revealed that keratinocytes and endothelial cells were the main source of TSP-1 in ACD although some inflammatory cells were also positive. To functionally characterize the role of TSP-1 in inflammation, we induced delayed-type hypersensitivity reactions by topical application of oxazolone to the skin of mice with targeted epidermal TSP-1 overexpression, in TSP-1-deficient mice and in wildtype mice. We found decreased edema formation in the inflamed ears of TSP-1 transgenic mice associated with a significant decrease in the number of enlarged blood vessels when compared to wildtype littermates. Conversely, TSP-1-deficient mice exhibited a persistent reaction, characterized by a delayed resolution of the inflammation and enhanced vascular remodeling, when compared to wildtype mice. Our data indicate that TSP-1 suppressed vascular remodeling and vascular leakage during an ACD and thereby decreased the extent of the experimental cutaneous inflammation, probably by downmodulating the effects of pro-angiogenic factors such as vascular endothelial growth factor A and by aiding in the resolution phase of the inflammation.

203 [Oral 036]

VEGF-A Plays a Key Role in the Induction of Chronic Inflammation and the Associated Lymphangiogenic Response

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Vascular endothelial growth factor-A (VEGF-A) expression is upregulated in several inflammatory diseases including psoriasis, delayed-type hypersensitivity (DTH) reactions and rheumatoid arthritis. To directly characterize the biological function of VEGF-A in inflammation, we evaluated experimental DTH reactions induced in the ear skin of transgenic mice that overexpress VEGF-A specifically in the epidermis. VEGF-A transgenic mice underwent a significantly increased inflammatory response that persisted for more than 1 month, whereas inflammation returned to baseline levels within 7 days in wild-type mice. Inflammatory lesions in VEGF-A transgenic mice closely resembled human psoriasis and were characterized by epidermal hyperplasia, impaired epidermal differentiation and accumulation of dermal CD4-positive T-lymphocytes and of epidermal CD8-positive lymphocytes. Surprisingly, VEGF-A also promoted lymphatic vessel enlargement, which might contribute to the increased inflammatory response, as lymphatic vessel enlargement was also detected in human psoriatic skin lesions. Combined systemic treatment with blocking antibodies against VEGF receptor-1 (VEGFR-1) and VEGFR-2 led to a decrease in lymphatic vessel size and potently inhibited inflammation. Together, these findings reveal a central role of VEGF-A in promoting lymphatic enlargement, vascular hyperpermeability and leukocyte recruitment, thereby leading to persistent chronic inflammation. They also indicate that inhibition of VEGF-A bioactivity might be a new approach to anti-inflammatory therapy.

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Induction of the Skin-Derived Antimicrobial Peptide Human Beta-Defensin-2 via NOD2/CARD15

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Human skin protects itself by the release of inducible antimicrobial peptides like the human beta-defensins hBD-2 and hBD-3. These beta-defensins are induced in keratinocytes upon contact with bacteria. Nothing is known about the responsible cellular receptors involved in the bacterial mediated induction of human beta-defensins in keratinocytes. Recently, a novel intracellular signal transduction molecule termed NOD2 (CARD15) has been discovered. NOD2 is composed of two N-terminal caspase recruitment domains, a nucleotide-binding domain and multiple C-terminal leucine-rich repeats which are responsible for the recognition of bacterial products. Recently, naturally occurring peptidoglycan fragments (i.e. muramyl dipeptide, MDP) were identified as the microbial motifs sensed by NOD2. We hypothesized that NOD2 might play a role in the chemical defense system of human skin. Therefore we investigated the gene expression of NOD2 in keratinocytes using real-time PCR. Furthermore, we cloned the NOD2 gene in an expression plasmid and used this construct to analyze the function of NOD2. Luciferase gene reporter assays were used to investigate the influence of NOD2 on hBD-2 promoter activation. Our results revealed that NOD2 mRNA is expressed in foreskin-derived primary keratinocytes. Overexpression of NOD2 in keratinocytes activated the transcription factor NF-kappaB as well as the hBD-2 promoter. Induction of hBD-2 through the NOD2 ligand MDP was increased in NOD2 overexpressing cells. Luciferase gene reporter analyses and site directed mutagenesis experiments demonstrated that functional binding sites for NF-kappaB and AP-1 in the hBD-2 promoter are required for NOD2-mediated activation of the hBD-2 promoter upon MDP stimulation. Mutation of the proximal NF-kappaB binding site in the hBD-2 promoter almost completely inhibited NOD2-mediated hBD-2 promoter activation. Our data indicate that NOD2 could serve as in intracellular receptor for bacterial ligands in keratinocytes. After activation by bacterial ligands NOD2 mediates the expression of inducible antimicrobial peptides like the human beta-defensin-2. This is the first report demonstrating that NOD2 mediates the inducible expression of a member of the human beta-defensin family.

205 [Oral 027]

Recombinant Eucaryotic Osteopontin (OPN) Induces Dendritic Cell Migration and Activation: Distinct Effects of Full Length OPN, N- and C-Terminal Fragments

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Osteopontin (OPN) is a secreted acidic, phosphoglycoprotein with a central GRGDS integrin binding domain. We have previously shown that OPN mediates chemotactic migration of Langerhans-/Dendritic Cells (LC/DC) to lymphnodes, thereby playing a central role during the sensitization phase of allergic contact hypersensitivity. Thrombin cleavage of OPN, close to the RGD sequence, results in two fragments of similar size but with distinct biological functions. The N-terminal fragment contains the αv binding RGD sequence, while the C-terminal fragment contains the predicted CD44 binding domain. OPN has a modular structure with multiple functional domains and its biological activity is influenced by its state of glycosylation and phosphorylation. *In vivo*, OPN fragments cleaved at inflammatory sites may influence DC-activation and migration. Here we investigated the secondary structure of recombinant eucaryotic OPN and compared its biological activity with thrombin cleaved fragments, neuraminidase treated OPN and a procaryotic OPN-GST-protein in its capacity to induce DC activation and migration. 6xHistidin(His_6)-tagged recombinant full length mOPN was expressed by stable transfected human HEK-293-EBNA cells and mOPN was purified by its His_6-tag. Full-length mOPN was cleaved by thrombin and fragments were separated and purified by N-terminal His_6-tag. Sialic acid moieties were removed by neuraminidase. Analyzing the circular dichroism of each protein, we found the eucaryotic protein and its fragments to be unstructured in solution showing a spectrum characteristic for a random coil with a minimum at 200 nm, which is in accordance to findings for OPN expressed in E. coli. Investigating the function of the full length eucaryotic OPN, compared to E. coli derived GST-OPN, we found both forms to induce DC activation, as measured by their expression of MHC-II and CD86. Interestingly, the DC activating effect of eucaryotic OPN is more pronounced than the effect of bacteria derived GST-OPN. Chemotaxis assays with immature BM-DC were performed with full length OPN and the generated fragments demonstrated the eucaryotic OPN to be the more potent chemotaxin. The most important domain mediating DC-migration seems to be located within the C-terminal half of the molecule, which contains the CD44 binding domain, because the N-terminal fragment, with the RGD-sequence, was a less effective chemoattractant. Neuraminidase treatment did not alter OPN activity, indicating that the promigratory function of OPN on DC is not influenced by Sialic acid residues. In conclusion we found eucaryotic OPN to have a random coil structure in solution. Functionally, OPN cleavage, *in vivo* occurring at sites of inflammation may influence the outcome of an immune response by differentially modulating DC activation and migration.

206 [Oral 055]

Functional Analysis of the Suppressor of Cytokine Signaling 1 Promoter in Human Keratinocytes

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We have previously shown that suppressors of cytokine signaling (SOCS) 1 is up-regulated by IFN-γ in normal human keratinocytes and is highly expressed in the epidermis of psoriasis and allergic contact dermatitis lesions. SOCS1 overexpression inhibited IFN-γ signaling in keratinocytes by reducing IFN-γ Rα phosphorylation and STAT1/3 activation. Forced expression of SOCS1 impaired IFN-γ-mediated membrane expression of ICAM-1 and HLA-DR, and the release of CCL2, CXCL9 and CXCL10 by keratinocytes. In this study we investigated SOCS1 promoter functionality in keratinocytes obtained from healthy subjects and patients affected by psoriasis, and its response to IFN-γ. The 5′ genomic region of human SOCS1 was cloned and reporter constructs bearing the $-1950/+297$, $-1620/+297$, $-819/+297$, $-540/+297$, $-248/+297$, $-27/+297$ and $-1950/+31$ promoter regions upstream of the luciferase gene were prepared. In transiently transfected keratinocytes, derived either from healthy or psoriatic donors, the deletion of promoter regions upstream of -248 and downstream of $+31$ resulted in a stonger luciferase activity, suggesting that these sequences contain binding sites for functional repressors. Putative binding sites for the SOCS1 transcriptional repressor Growth Factor Indipendence-1 (GFI-1) were found in regions between -1620 and -819, -819 and -540, -540 and -248, and $+31$ and $+297$. However, EMSA analyses performed with these fragments and keratinocyte protein extracts in the presence of an anti-GFI-1 antibody or molar excess of oligonucleotides containing wild-type or mutated GFI-1 binding sites revealed that GFI-1 binds efficiently only to its consensus sequence in $-819/-540$ and $-248/+31$ regions. Basal luciferase activity was increased by 3 folds in IFN-γ-treated keratinocytes when the full SOCS1 promoter ($-1950/+297$) was used. Progressive 5′ deletion of the SOCS1 promoter did not significantly alter responsiveness to IFN-γ, although we could detect different gel-retarded complexes in EMSA when nuclear extract from unstimulated and IFN-γ-treated keratinocytes were employed with the $-540/-248$ fragment. RNase protection experiments demonstrated that SOCS1 mRNA expresson was decreased in psoriatic keratinocytes compared to cells from healthy controls, and this decrement depended on a reduced SOCS1 promoter activity, as assessed in transiently transfected cells. Reducing GFI-1 binding to the SOCS1 promoter can lead to upregulation of SOCS1 gene transcription and thus prevent the expression of inflammatory genes in IFN-γ activated keratinocytes.

207 [Oral 061]

Analysis of Antimicrobial Activity and Gene Regulation of the Skin-Derived RNase 7

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Healthy human skin is constantly exposed to various microorganisms, but usually not infected. One reason for this high natural resistance might be the production and release of endogenous antimicrobial proteins by keratinocytes. Recently, we isolated the antimicrobial protein RNase 7 from stratum corneum extracts. The aim of this study was to explore the role of RNase 7 in the chemical defence system of human skin. We analysed its antimicrobial activity and studied its gene regulation in primary keratinocytes. For investigation of the antimicrobial activity we used RNase 7 isolated from stratum corneum as well as recombinant RNase 7 expressed as a fusion-protein in *E. coli*. RNase 7 exhibits a broad spectrum of high antimicrobial activity against many pathogenic Gram-negative and Gram-positive bacteria including Vancomycin-resistant *Enterococcus faecium*, *Enterococcus faecalis* and MRSA. Deletion of the ribonuclease activity of RNase 7 by exchange of two amino acids in the active center of the enzyme did not reduce its antimicrobial activity against *E. coli* suggesting that the ribonuclease activity is not essential for killing of *E. coli*. Unlike the inducible human beta-defensins hBD-2 and hBD-3, RNase 7 is expressed in normal skin at a very high level (approximately 100–400 μg RNase 7 were purified from 50 g stratum corneum extracts). Real-time RT-PCR revealed high constitutive gene expression of RNase 7 in primary keratinocytes. The high gene expression of RNase 7 requires the single intron located in the 5′-UTR as shown by Luciferase gene reporter assays. Treatment of the cells with supernatants of adherent cultures of *P. aeruginosa* and *S. aureus* further increased expression of RNase 7 in primary keratinocytes (up to 50-fold induction) as well as treatment with 50 ng/ml phorbol-myristate-acetate (100-fold induction). Interestingly, gene induction was almost completely inhibited by co-treatment with all-trans retinoic acid. Taken together the high expression of RNase 7 in keratinocytes combined with its high antimicrobial activity suggests, that RNase 7 might play an important role in the defence of the skin against various pathogenic bacteria.

208 [Oral 041]

The Proteasome Inhibitor bortezomib (PS-341) Reverses Constitutive Activation of NF-kappaB Pathway and Promotes Apoptosis in Human Cutaneous T Cell Lymphoma (CTCL)

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Advanced stages of CTCL are associated with a poor response to death-inducing chemotherapeutic agents, including mitochondrial drugs. Since we previously demonstrated that an activation of the nuclear Factor-kappaB (NF-κB) pathway plays a key role in this resistance, we investigated the effects of bortezomib (PS-341), an inhibitor of the proteasome which has shown potent anti-tumor properties. We evaluated the effects of PS-341 (5 to 50 nM) on the viability of CTCL cell lines (HUT78, SeAx, Myla) lines and tumor cells from patients with Sezary syndrome (SS). The modifications of NF-κB activity were studied by electrophoretic mobility shift assay (EMSA). The cellular localization of NF-κB proteins was analysed by immunocytochemistry and confocal microscopy. The expression of cytoplasmic NF-κB inhibitors (I-κB) and of molecules of the Bcl-2 family was investigated by immunoblotting.

Results demonstrate that PS-341 almost completely reverses the constitutive nuclear translocation of NF-κB, as shown by EMSA and cytochemistry, in a dose- and time-dependent fashion. In both CTCL cell-lines and tumor cells from SS patients, inhibition of constitutive NF-κB by bortezomib led to rapid induction of apoptosis, as shown by annexin V/propidium iodide staining and mitochondrial transmembrane potential alterations, as well as by immunoblot analysis of procaspase 3 and of poly(ADP-ribose)polymerase cleavage. Immunoblotting analysis revealed that bortezomib-induced apoptosis of CTCL cells was associated with an up regulation of the pro-apoptotic Bax member of the bcl-2 family, while expression of contra-apoptotic molecules such as Bcl-x(L) or Bcl-2 was not altered.

These results demonstrate that bortezomib (PS-341) is able to reverse *in vitro* the constitutive activation of NF-κB, and to induce apoptosis in CTCL cells. This set of data warrants further studies investigating *in vivo* the effects of bortezomib in CTCL patients showing resistance to classical systemic treatments.

209 [Oral 005]

Interleukin 4 Promotes TC1-Driven, Protective Anti-Cancer Immunity

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Identifying agents that boost antigen-specific immune responses is an important goal of researchers involved in cancer immunotherapy. Recently, we found that the co-administration of the polycation poly-L-arginine (pR) and βgalactosidase (βgal; thereafter referred to as pR-PV) yields substantially higher numbers of specific, IFNγ-secreting, $CD8^+$ T cells than s.c. βgal-application. In addition, there are studies showing that IL-4 is capable of promoting the activation of TH1 lymphocytes provided that it is applied early after the induction of the immune response and at high doses. Based on these observations, we asked whether IL-4 can be used to augment the efficacy of the pR-PV. To test this hypothesis, BALB/c mice were injected s.c. with the pR-PV either alone or admixed with IL-4. Seven days later, CD8 + T cells producing either IFNγ or IL-4 in response to βgal were quantified by ELISPOT analysis. Results obtained showed that co-administration of IL-4 significantly enhances the number of vaccine-induced, specific $CD8^+$ T lymphocytes producing IFNγ. In addition, we found the IL-4 treatment used to shift the specific immune response towards a TC1 phenotype. To investigate the effect of IL-4 administration on the clinical efficacy of the pR-PV, BALB/c mice were injected on days 0 and 14 with pR-PV +/− IL-4 and challenged on day 24 by the s.c. inoculation βgal-expressing RENCA cells. None of the naive controls (n = 6) rejected the tumor inoculum. S.c. administration of the pR-PV protected 3/6 (50%) animals. The highest protection rate (5/6; 83%) was obtained by the combined use of the pR-PV and IL-4.

Together, these results demonstrate that IL-4 has the potential to act as a natural adjuvant capable of inducing a protective TC1-driven anti-cancer immune response.

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Sex Hormones Modulate Interleukin-5 Production By Human Skin Mast Cells With Gender Specific Differences

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Mast cells (MC) are localized in close vicinity to blood vessels and thereby exposed to high levels of serum derived factors. We previously reported on differences between (female) breast skin derived human MC (bsMC) as opposed to (male) foreskin derived MC (fsMC) in terms of cytokine production. In the present study, we hypothesized that MC may be significant targets of sex steroid action and that distinct hormonal networks may give rise to divergent cytokine production by fsMC and bsMC. We also sought to clarify if responsiveness to hormones differed among MC subsets. MC purified to homogeneity from both skin compartments were first tested for the expression of sex steroid receptor transcripts by semiquantitative RT-PCR analysis. MC expressed mRNA for androgen receptor (AR), estrogen receptor (ER)α, ERβ, and progesterone receptor (PR). The levels detected in MC were higher than those found in other primary haematopoietic cells (PBL, monocytes, granulocytes), with PR expression being even exclusively confined to MC. While levels of PR and ERβ displayed an even distribution among fsMC and bsMC, substantially higher levels of ERα were associated with bsMC. Conversely, AR specific transcript was preferentially expressed by fsMC. To study a possible impact on MC cytokine production, both bsMC and fsMC were pre-cultured for two weeks with stem cell factor (100 ng/ml) + interleukin (IL-)4 either in the absence or presence of progesterone (P, 1 μM), dihydrotestosterone (T, 500 nM), or 17 β-estradiol (E, 200 nM), primed with human IgE, washed extensively and challenged by anti-IgE at 1×10^6/ml. The resulting supernatants were analyzed by ELISA for IL-1β and IL-5 levels. While IL-1β did not appear subject to regulation by sex hormones, IL-5 was substantially reduced by P from 190.4 ± 50.4 to 104 ± 33.8 ng/ml/10^6 cells (n = 6, $p < 0.02$). Interestingly, despite the presence of similar levels of PR in both MC subsets, the downregulatory effect of P was strictly confined to bsMC. On the other hand, only fsMC showed enhanced IL-5 production following T treatment from 260.4 ± 23.2 to 395.9 ± 35.6 ng/ml/10^6 cells (n = 4, $p < 0.02$). We conclude that MC in human skin are equipped with the prerequisites to respond to sex steroids, that MC responses to these hormones display gender specific differences, and that IL-5 production by MC may be favored by a "male", and concomitantly inhibited by a rather "female" environment.

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Evidence for a Restricted Rather Than Generalized Stimulatory Response Of Skin-Derived Human Mast Cells Towards Substance P

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The neuropeptide substance P (SP) is believed to impact mast cell (MC) function thereby contributing to the bidirectional communication between MC and nerves. However, MC heterogeneity is a well-known phenomenon that comprises species-, tissue-, and differentiation-stage dependent differences among individual MC populations. Having developed a technique to purify MC to homogeneity from human breast skin tissue, we analyzed the direct effects of SP on degranulation and cytokine production of this MC subset. As expected, SP dose-dependently induced histamine release, the extent of which (at an SP dose of 30 μM) was only slightly lower than that induced by immunological stimulation via the high affinity receptor for IgE (FcεRI). When the two activation pathways were compared directly for the production of proinflammatotory cytokines, both analogous and divergent effects were found. Only the cross-linking of FcεRI was able to trigger high-level TNF-α secretion (100.1 ± 28.5 pg/10^6 cells and ml after 24 h versus 10.1 ± 0.8 at baseline, n = 8; $p < 0.02$) and low (but significant) IL-8 increase (5.01 ± 1.26 ng/10^6 cells and ml after 24 h versus 4.36 ± 1.23 at baseline, n = 8; $p < 0.01$), while SP (at 0.3–30 μM) remained without any effect. Likewise, SP (at 30 μM) had no additional effect on these two cytokines when combined with anti-IgE. We have shown previously that FcεRI crosslinking suppresses the levels of IL-6 accumulating in the supernatant of human skin MC. Interestingly, SP displayed an even higher suppressive effect in this regard (31.2 ± 12.8 pg/10^6 cells and ml after 24 h with SP at 30 μM versus 48.0 ± 12.9 at baseline, n = 7; $p < 0.005$). No additional effect from anti-IgE was observed when combined with SP. To elucidate whether the decreased IL-6 concentration in the supernatant was driven by reduced levels of IL-6 specific mRNA, a quantitative real time PCR technique using the SYBRGreen system was utilized. The amount of mRNA copy numbers was assessed as (each per 25 ng total RNA): 2178 for IL-6 (baseline) versus 2307 (SP). Likewise, SP had no effect on the concentration of either IL-8 (2665/control versus 2461/SP) or TNF-α transcript (94/control versus 72/SP). This suggests that the downregulation of IL-6 in the supernatant proceeds through a post-transcriptional mechanism that may involve the recently described degradation of this cytokine by MC tryptase released from MC granules on SP mediated degranulation. We conclude that SP acts on human skin MC in a very selective manner by eliciting degranulation but without the induction of proinflammatory cytokines. In addition, our data underline that IL-6 availability in the vicinity of human MC can be regulated by mechanisms other than transcription.

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Papillomavirus-like Particle-Based Amyloid Beta Vaccine Induces Protective Autoantibody Response

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Non-infectious virus-like particles (VLP) composed of major capsid protein L1 are successful as vaccine to prevent human papillomavirus (HPV) infection and associated neoplasia. In addition, chimeric VLP have been generated that incorporate foreign or self-antigens, and immunizations have induced high levels of (auto-)antibodies (ab), whereas immunizations with linear peptides were less effective. Ab were long-lasting and functionally active in experimental animal models.

The neuro-degenerative disorder Alzheimer's disease (AD), is characterized by neurofibrillary tangles and amyloid plaques, consisting of amyloid-beta (Abeta) fibrils. There is no effective therapy to prevent/cure AD. Recent studies have focused on anti-Abeta ab to inhibit fibril formation and/or to resolve Abeta-aggregates.

Chimeric bovine papillomavirus (BPV1) L1 was generated by inserting 9 amino acid (aa) N-terminal peptide of Abeta into an immunogenic VLP surface loop (Abeta-VLP) and expressed in Sf-9 cells. Electron micrographs demonstrated efficient self-assembly into capsomeric VLP. Antigenicity of the inserted Abeta epitope was verified by Western blot. This peptide shows aa sequence identity in human and rabbit representing a self-antigen for both species. To examine whether Abeta-VLP are capable to break B-cell tolerance, NZW rabbits were immunized with Abeta-VLP using Freunds adjuvant. After five months no signs of toxicity were observed. By ELISA, rabbit inoculated with Abeta-VLP developed strong ab response (titer > 10.000) to Abeta peptide 1-9, whereas control rabbit did not. Reactivity was specific for the Abeta epitope and blocked by homologous, but not control peptide. Immune sera revealed a weak but significant neuroprotective effect against pre-aggregated amyloid fibers and recognized amyloid plaques in post mortem brains sections of AD patients.

These data support VLP as a tool to overcome tolerance to self-antigens with potential of inducing therapeutically useful auto-ab responses in humans. This hypothesis is currently evaluated by *in vivo* models of AD.

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CCL27-CCR10 Interactions Play an Important Role in Skin-Associated T Cell Priming

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The skin immune system provides essential sentinel functions at the interface between the environment and the organism. Next to infectious agents, increasing numbers of xenobiotics are constantly screened by cellular members of the skin immune system. After recognition, processing, presentation and subsequent T cell priming, the recruitment of memory T cells to the skin represents a critical step during the elicitation of effective immune responses against foreign antigens. Recently, we identified the novel skin-specific chemokine CCL27 as well as its receptor CCR10 and demonstrated that CCL27-CCR10 interactions regulate the recruitment of antigen-specific memory T cells during the elicitation phase of hapten-induced contact hypersensitivity. In the present study, we investigated the role of CCL27-CCR10 interactions during the sensitization phase of contact hypersensitivity. During the induction phase of hapten-induced contact hypersensitivity neutralization of mCCL27 significantly suppressed subsequent DNFB-induced elicitation responses. Re-challenge as well as adoptive transfer experiments indicated that the blockade of CCL27-CCR10 interactions significantly impaired the generation of hapten-specific memory T cells *in vivo*. Taken together, findings of the present study suggest that chemokine ligand-receptor interactions also play an important role in the afferent phase of immune responses.

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UV-Injury, Apoptosis, Chemokines and Leukocyte Recruitment: An Amplification Cycle Triggering Cutaneous Lupus Erythematosus

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Lupus erythematosus (LE) is an autoimmune disease demonstrating photosensitivity, increased apoptosis, and cutaneous leukocyte recruitment. In the present study, we identify the CXCR3 ligands, CXCL9 (*MIG*), CXCL10 (*IP-10*), CXCL11 (*I-TAC*) being the most abundantly expressed chemokine family members (n = 42) in cutaneous LE. Expression of these ligands corresponded with a marked inflammatory infiltrate consisting of mainly CXCR3-positive cells including CLA + lymphocytes and CD123 + /BDCA2 + plasmacytoid dendritic cells (pDC). Circulating pDCs express CCR5, CXCR3, and CXCR4 and produce high levels of type I interferon as well as chemokines after stimulation with either virus, apoptotic bodies or immunostimulatory DNA sequences (ISS). Type I interferons, in turn, are potent inducers of CXCR3 ligands in cellular constituents of the skin such as keratinocytes, dermal fibroblasts and endothelial cells. Furthermore, we show that UVB-irradiation induces a distinct set of chemokines in keratinocytes including CCL5, CCL20, and CCL27. Taken together our data suggest an amplification cycle with UV-injury inducing apoptosis and chemokine production which in turn mediate the recruitment and activation of lymphocytes and IFN–producing pDCs which subsequently release more effector cytokines and chemokines finally leading to the development of cutaneous LE lesions.

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Synergistic Activation of the Chemokine CCL20/MIP-3α Expression in Dermal Cells by Pro-Inflammatory Cytokines is Dependent on Activation of the Transcription Factors NF-κB p65 and p52, as well as Stat1α

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The inducible CC chemokine CCL20/Macrophage Inflammatory Protein (MIP)-3α is over-expressed in psoriatic lesions. Primary keratinocytes (KC), dermal fibroblasts (HDF), microvascular endothelial cells and dendritic cells produce MIP-3α upon stimulation with, e.g. TNF-α/IL-1β, IFN-γ or IL-17, which are also overexpressed in psoriasis. We compared the effects of TNF-β, IFN-β and IL-17 alone or in combination in primary human KC and HDF, as well as keratinocyte and fibroblast cell lines (HaCaT, A431, 1BR3GN, HT1080). Primary KC and HDF showed a synergistic induction of CCL20 in the presence of TNF-α and IFN-γ or IL-17, with the highest levels reached by the combination of these three cytokines. The same pattern was observed also in 1BR3GN and HT1080 cells, but not in HaCaT and A431. For the investigation of signal transduction pathways involved in this synergistic activation, we chose HT1080 cells as a more convenient system than primary cells. The upregulation of CCL20 at both mRNA and protein levels required between 6 and 24 hours of incubation. IL-17 alone had no effect on any of the signalling pathways investigated (NF-κB, MEK1/2, MKK4, MKK3/6, p38 MAPK, Stat proteins 1-6). TNF-α induced the activation of NF-κB p65, and IFN-γ activated Stat-1α. The combination of all three cytokines, in addition, led to an upregulation of NF-κB p100/p52 mRNA and nuclear translocation of p52. Transfection of siRNAs directed against NF-κB p65, NF-κB p100/p52 and Stat1 into HT1080 cells significantly inhibited CCL20 expression after stimulation with the triple cytokine cocktail. These three signal transduction mediators are thus involved in the synergistic activation of CCL20 in HT1080 cells and, given the similarity of expression patterns, potentially also in primary KC and HDF.

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Kinetic Study Of T Cells Phenotype In The Drug Hypersensitivity Syndrome

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The drug hypersensitivity syndrome (DHS) or Dress (Drug reaction with eosinophilia and systemic symptoms) is a severe toxic reaction associated with visceral manifestations sometimes leading to death. Medications involved are mostly antiepileptics. Clonal T cell expansions are found in 40% of patients.

Sixteen patients with DHS were included in our study. CD4 + and CD8 + T cells were separated from PBMC using negative selection on MACS columns. A T lymphocyte repertoire analysis was performed with the immunoscope technique using Vβ and Cβ specific primers. Lymphocyte phenotype (activation markers: CD25, CD68, CD134, CD45RO, HLA DR, FasL; cutaneous homing receptors: CLA, CCR4) and cytokine secretion (IL2, IL5, IFNγ, TNFα, IL4, IL8, IL13 and IL10) were analysed with a flow cytometer on blood samples collected at D5, D30, D90, D180 and D360. When immunoscope analysis showed a repertoire bias (i.e. non Gaussian) with an expanded lymphocyte population within a particular Vβ family, a flow cytometry analysis with an anti-Vβ antibody was performed in a second time to characterize the phenotype and the cytokine secretion profile of the expanded population. In order to study the modifications of gene expression in DHS, we performed a DNA array of 901 genes expressed in PBMC for 3 patients with DHS secondary to carbamazepine and minocycline.

In our multicentric and prospective study, we found that T cells presented an activated phenotype, more remarkable within the CD8 + subset. CD4 + and CD8 + cells overexpress cutaneous homing markers at the early stages of the disease. The cytokine profiling of CD4 + T cells is Th1 orientated, the proportion of CD4 + and CD8 + T cells secreting IFNγ and TNFα increases between D5 and D90 which may be an explanation for relapses when corticotherapy is decreased in this period. The analysis of expanded populations with anti-Vβ antibody showed that these cells express higher levels of activation markers and secrete higher levels of IFNγ, TNFα and IL2. The gene expression analysis on CD4 + and CD8 + populations confirms the results obtained by flow cytometry analysis and more generally shows overexpression of genes involved in cellular activation, apoptosis, cutaneous homing and production of proinflammatory cytokines.

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TLR2 and TLR4 Mediate the Recognition of Pathogens in Vaginal Epithelial Cells

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Epithelial cells of the vagina are important in providing protection against various pathogenic microorganisms, e.g. *Candida albicans*. Vaginal epithelial cells produce proinflammatory cytokines and antimicrobial peptides, which mediate the killing of pathogenic fungi. Toll-like receptors (TLRs) are known to be important in mediating mechanisms of innate host defence. We aimed to explore whether TLR signalling pathway and activation of proinfalmmatory cytokines and antimicrobial peptides could be responsible for the protection against pathogenic microorganisms by vaginal epithelial cells.

TLR expression on vaginal sections was detected by imunohistochemical staining with specific antibodies. Quantitative real time RT-PCR, flow cytometry and Western-blot analyses were used to detect the expressions of TLR2 and TLR4 in immortalized vaginal epithelial cell line (PK cells). Activation of NF-κB transcription factor in PK cell after challenge with microbial products was examined using ELISA-based TransAM™ assays. Quantitative real-time RT-PCR was used to examine the expressions of pro-inflammatory cytokines (IL-8, TNFα, IL-1α and the inducible human antimicrobial peptide (β-defensin 2)) after incubation with heat-killed *Candida albicans,* zymosan, LPS and PGN. ELISA assay and fluorescent immunostaining were used for detection of cytokines and human β-defensin 2 (hBD2) produced by vaginal epithelial cells.

We found that TLR2 and TLR4 receptors are expressed *in vivo* in vaginal epithelia and *in vitro* in PK vaginal epithelial cell line. Quantitative real time PCR showed that IL-8, TNFα and hBD2 mRNAs were significantly ($p < 0.05$) up-regulated in PK cells 3 h after treating them with LPS, PGN and *Candida*. Although we found that NF-κB had a constitutive activity in PK cells, NFκB assay showed that LPS and PGN can further induce this transcription factor. In good correlation with the results of real-time RT-PCRs, ELISA assays and immunostainings showed that microbial compounds markedly induced the production of proinflammatory cytokines and antimicrobial peptides in PK cells 12 and 24 h after treatment. In our work, we give an evidence that recognition of pathogens by vaginal epithelial cells results in the production and release of pro-inflammatory cytokines and antimicrobial peptides, which might lead to the elimination of pathogens from the vaginal tract and activation of both innate and acquired immune responses.

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Induction of Proinflammatory Cytokines and RNase 7 by *Staphylococcus Aureus* in Human Keratinocytes

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The gram-positive bacterium *Staphylococcus aureus* is a major cause for a variety of skin infections. A proinflammatory response against *S. aureus* is described in human keratinocytes, but little is known about the inducing pathogen-associated molecules (PAMs) involved and their mode of activation. To analyse soluble PAMs released by *S. aureus*, we investigated the supernatants of different strains cultured under various conditions for their potential to induce proinflammatory cytokines and antimicrobial peptides in the human keratinocyte cell line HaCaT as well as in primary keratinocytes. By Realtime-PCR analyses we found that supernatants of different *S. aureus*-strains induced the proinflammatory cytokines IL-8 and TNF-alpha and the antimicrobial protein RNase 7, but not human beta defensin (HBD)-2. Supernatants of static or suspension bacterial cultures always resulted in strong IL-8 induction. Interestingly, only supernatants of bacteria of 4–5 day cultivation under static conditions, which corresponded to the late exponential growth phase, yielded high induction of the *S. aureus*-killing RNase 7 and TNF-alpha as well as IL-8.

Partial purification and biochemical characterization experiments by size exclusion- and ion-exchange-HPLC indicated that the *S. aureus*-derived RNase 7-inducer, which appears to be anionic eluting upon size exclusion-HPLC in the void-volume, also induces IL-8, but not HBD-2, an antimicrobial peptide that is not active against *S. aureus*.

Since toll-like receptors (TLRs) are important PAM-receptors and recognition through TLR2 was described as a major pathway for the activation of epithelial cells by staphylococci, we over expressed TLR2 in HaCaT cells and primary keratinocytes prior to stimulation with *S. aureus* supernatants. In both cases we found no increase of IL-8 production of cells over expressing TLR2, indicating that TLR2 is not involved in the induction of IL-8 by soluble PAMs produced by *S. aureus* in the late exponential phase under static conditions. We conclude from these findings that – depending on culture conditions – *S. aureus* secretes PAMs that can be recognised by skin keratinocytes leading to a rapid innate epithelial defense reaction through induction of the staphylocidal protein RNase 7 and proinflammatory cytokines. The mechanism of induction is not clear yet, but does not seem to involve TLR2.

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Inhibition of *Pseudomonas Aeruginosa*-Induced Human Beta-Defensin-2 Expression by the Th2 Cytokines IL-4, IL-10 and IL-13 in Keratinocytes

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Skin represents the first barrier in the defence system of human beings. Antimicrobial peptides are part of this innate immune system. In healthy skin the concentrations of these antimicrobial peptides are low but accumulate at sites of infection. Atopic dermatitis (AD) is a common chronic inflammatory skin disorder. Bacterial or viral superinfection more often occurs in patients with AD as compared to patients with Psoriasis. A recently published study has shown that levels of Th2 cytokines like IL-4, IL-10 and IL-13 are elevated and levels of antimicrobial proteins like human beta-defensin-2 (hBD-2) are decreased in the skin of patients with AD. The expression of hBD-2 in keratinocytes is strongly induced by bacteria like *Pseudomonas aeruginosa* (PA) leading to the hypothesis that the bacteria-mediated induction of hBD-2 (and other inducible antimicrobial peptides) might be disturbed in AD patients. Therefore, we investigated whether the PA-induced expression of hBD-2 in keratinocytes is inhibited by the Th2 cytokines IL-4, IL-10 and IL-13. HaCaT and primary keratinocytes were preincubated for 6 h with 40 ng/ml of IL-4, IL-10 or IL-13, respectively, or a combination of 40 ng/ml IL-4 and 40 ng/ml IL-13 and subsequently treated with PA supernatans for 15 h. The hBD-2 expression was analysed using real-time PCR, luciferase gene reporter assays and enzyme-linked immuno sorbent assay (ELISA). Stimulation of keratinocytes with PA supernatants resulted in a strong induction of hBD-2 which was significantly decreased in the presence of some Th2 cytokines. IL-4 was found to be the strongest inhibitor (decrease of hBD-2 induction by 80%–90%) followed by IL-13 (decrease of hBD-2 induction by 70%–80%) and IL-10 (decrease of hBD-2 induction by 20%–30%). Treatment using a combination of IL-4 and IL-13 inhibited hBD-2 induction by 70%–80%. These results suggest that the low levels of antimicrobial proteins in the skin of AD patients might be caused by the high levels of Th2 cytokines which strongly inhibit the bacteria-mediated induction of antimicrobial proteins in keratinocytes. This might be one reason for the susceptibility of AD patients to bacterial and viral superinfections and could lead to a new clinical relevant view in preventing superinfections of patients with AD.

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Fumaric Acid Esters (FAEs) Mediate their *in vitro* Immunosuppressive Effects by Glutathione (GSH) Depletion and Induction of Heme Oxygenase (HO-1)

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Mixtures of FAEs are the most commonly used, orally applied systemic therapeutics for psoriasis in Germany. Despite their clinical success, the molecular mechanisms of FAEs have been not defined yet. In this study, we characterized the *in vitro* immunosuppressive activity and mechanism of FAEs in primary human immune cells. Dimethyl fumarate (DMF) and diethyl fumarate (DEF) [but not monomethyl fumarate (MHF), monoethyl fumarate (MEF), and fumaric acid (FA)] exhibited potent depression of LPS-induced monokine secretion (TNF-alpha, IL-1beta, IL-12) and PHA-triggered IFN-gamma secretion in hPBMC. Moreover, only DMF and DEF were active in inhibiting T-cell proliferation after mitogen stimulation and in mixed lymphocyte reaction (MLR). Since DMF had been described to induce oxidative stress via depletion of reduced glutathione (GSH), we tested the expression of the potent anti-inflammatory stress protein HO-1. Remarkably, only DMF and DEF led to a strong and dose-dependent induction of HO-1 mRNA in monocytes. HO-1 induction was completely prevented by exogenous GSH supplementation. GSH substitution was also active in abolishing the immunosuppressive *in vitro* effects of DMF and DEF, i.e., the depression of immunostimulatory cytokine secretion and T-cell proliferation. In summary, the *in-vitro* immunosuppressive activity of DMF and DEF is mediated by a functional depletion of reduced GSH and is associated with a strong induction of the antiinflammatory stress protein HO-1. Further clarification of the mechanism of clinical successful FAEs may lead to new approaches for therapy of psoriasis and other inflammatory and immune diseases.

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The Expression of Leukocyte-Chemoattractive Factors Suggest a Pathophysiological Link Between Psoriatic Skin and Bone Disease

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Psoriasis is a chronic inflammatory disorder characterized by sterile infiltration of skin lesions with inflammatory cells, primarily T cells and neutrophils. Up to 35% of patients also suffer from concomitant joint disease. The similarities between skin and bone affection are particularly prominent in a variant of psoriatic arthritis called chronic recurrent multifocal osteomyelitis (CRMO). CRMO is typically associated with pustular psoriasis and presents as a chronic relapsing primary aseptic osteomyelitis characterized by infiltration of the metaphyses of tubular long bones, clavicles, or vertebrae with neutrophils and lymphocytes. The factors involved in the influx of inflammatory cells in CRMO have not yet been clearly identified.

Immunohistochemical and *in situ* hybridization studies of bone specimen obtained from a patient with CRMO revealed the expression of several leukocyte-chemoattractive factors including interleukin-8, interleukin (IL)-16 and lymphotactin (Ltn). While the expression of IL-16 and Ltn was largely confined to infiltrating T cells, osteoblasts were also identified as producers of chemotactic factors including IL-8 and MIP-3alpha. To further investigate the posssible role of osteoblasts as sources of chemokines in CRMO, primary osteoblast cultures ($n = 5$) were treated with TNF-α and IL-1β to mimick the proinflammatory environment present in psoriatic arthritis. Investigation by cDNA array and quantitative real-time RT-PCR techniques showed a strong induction of several chemokines, among them IL-8, MCP-1, MCP-2, MIP-3alpha, IP-10 and RANTES. The cytokine-induced production of these factors was confirmed on the protein level by ELISA, and could not be suppressed by co-incubation of osteoblasts with cyclosporin or azithromycin, two drugs used in the treatment of CRMO. Our study provides evidence for a role of leukocyte-chemoattractive factors in the pathogenesis of CRMO. Under the influence of pro-inflammatory cytokines, osteoblasts, in a certain analogy to the role of keratinocytes in psoriatic skin disease, may acquire non-professional immune functions and contribute to the formation of bone lesions in psoriasis.

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Expression and Activity of IL-17 in Cutaneous T Cell Lymphomas (CTCL)

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IL-17 is a pro-inflammatory cytokine mainly produced by activated CD4$^+$ memory helper T cells and we have previously shown in mice that IL-17 was able to act as a tumor growth-promoting or -inhibiting factor, depending on the model. In the present work, we investigated the expression and function of IL-17 in mycosis fungoides (MF) and Sezary syndrome (SS), two types of CTCL usually bearing a CD4$+$CD45RO$+$ memory helper T cell phenoype. Results from RT-PCR studies and from enzyme linked immunoassays demonstrate that CTCL cell lines derived from MF (Myla) and SS (SeAx), as well as freshly isolated circulating tumor cells from patients with SS, do express IL-17 mRNA and secrete this cytokine following stimulation with PMA (100 ng/ml) $+$ ionomycin (1 μg/ml) or anti-CD3 (1 μg/ml) $+$ anti-CD28 (5 μg/ml) antibodies. However, IL-17 does not act as a growth factor for MF or SS cell lines *in vitro*. In addition, five out of 10 MF/SS biopsies expressed IL-17 mRNA, while this cytokine was not detected in normal skin. In 2 patients with MF, IL-17 transcripts were not detected in initial biopsies, whereas an upregulation of this cytokine was demonstrated during progression of the disease. Furthermore, an association between IL-17 expression and polymorphonuclear neutrophil infiltration was recorded in cutaneous lesions from 5 MF/SS patients, including two cases presenting an unusual pustular form of CTCL. Results from RT-PCR studies of CTCL lesions, which revealed a coexpression of IL-17 with IL-8, support the contribution of IL-17 to the recruitment of neutrophils, most likely via a paracrine mechanism involving keratinocyte-released IL-8. This study is the first demonstration of IL-17 production by a human tumor, suggesting that synthesis of IL-17 within the tumor microenvironment alters the development of the inflammatory reaction and may influence tumor phenotype and growth.

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Human Leukocyte Elastase Induces Keratinocyte Proliferation by Epidermal Growth Factor Receptor Activation

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Epidermal hyperproliferation and neutrophil infiltration are major histopathological changes observed in psoriasis. Neutrophils contain human leukocyte elastase (HLE), which is released at sites of inflammation. Previous studies demonstrated that HLE is present in psoriatic lesions and induces keratinocyte hyperproliferation *in vitro* and *in vivo*. To determine the molecular mechanisms linking a proteolytic effect of HLE and epidermal hyperproliferation, we examined the effects of HLE-induced signaling in human keratinocytes. Application of 100 nM HLE resulted in a transient calcium influx in FURA2-loaded human HaCaT keratinocytes observed by single-cell fluorescence imaging. The calcium signal was concentration dependent and was inhibited by addition of the HLE inhibitors elafin and secretory leukocyte protease inhibitor (SLPI). The calcium signal was neither inhibited by pertussis toxin, cholera, or by pre-stimulation with trypsin. Incubation with the tyrosine kinase inhibitor genistein, a protein kinase C inhibitor (a myristoylated anti-epidermal growth factor receptor (EGFR) fragment), as well as incubation with neutralizing EGFR antibodies abolished the HLE-induced calcium influx. The supernatants of HLE-treated keratinocytes induced a calcium signal in separately cultured keratinocytes. This could be inhibited by the addition of anti-transforming growth factor alpha (TGF-alpha) antibodies. Application of HLE-induced keratinocyte proliferation, which could be inhibited by neutralizing of anti-EGFR and anti-TGF-alpha antibodies. Herein we demonstrate that HLE induces keratinocyte proliferation by proteolytic activation of an EGFR signaling cascade involving TGF-alpha.

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The Distribution of GITR and GITRL in Atopic Dermatitis, Suggest Inhibition of Regulatory T-Cells Locally in the Dermis

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Background. TARC (thymus and activation-regulated chemokine) is produced in the basal keratinocytes of affected skin in AD and induces chemotaxis of CCR4$+$ cells, thereby attracting skin homing lymphocytes. Recently, it has become clear that a defect in the development of CD4$+$CD25$+$ regulatory T-cells (Tregs) results in eczema. The Glucocorticoid-Induced Tumor necrosis factor Receptor family-related gene (GITR) is expressed on Tregs where binding to the ligand (GITRL) inhibits the function of Tregs. We therefore examined the plasma levels and localization of GITR and GITRL in AD and correlated this to TARC.

Methods. Heparinized blood samples were taken from 78 AD patients (43 female, age 17,6 year $\pm$ 14,1 year and 35 male age 21,3 year $\pm$ 15,8 year). The samples were centrifuged and the plasma was stored at -80°C. We measured the levels of TARC, GITR and GITRL in plasma, in duplicates, using a sandwich ELISA. Immunohistochemical localization of GITR and GITRL was done on biopsies from both involved and uninvolved skin. Values are expressed as median with 25%–75% range.

Results. Plasma levels of GITRL were not increased in AD patients (984 pg/ml $\pm$ 281 pg/ml – 1827 pg/ml) compared to normal healthy volunteers (873 pg/ml 457 pg/ml – 1730 pg/ml). $P = 0{,}831$. Neither were the soluble GITR, which inhibits GITRL, increased compared to normal healthy volunteers (104 pg/ml vs. 82 pg ml) $P = 0{,}149$. We measured TARC in the same samples, observing a significant correlation between high levels of TARC and high levels of GITRL ($r = 0{,}509$, $p = 0.01$).

By immunohistochemistry, cells expressing GITR and GITRL were localized to the dermis of AD patients, and never observed in the epidermis.

Conclusions: Patients with AD do not have increased levels of GITRL and of sGITR, but have accumulation of cells expressing both GITR and GITRL in the dermis, suggesting that local inhibition of Treg takes place in AD.

As Treg express CCR4, the receptor for TARC, and the fact that we observed a correlation between TARC and GITRL, suggest that TARC attracts the Tregs, but the Tregs may not be able to exert their local suppressive function on activated lymphocytes in the dermis, because they are blocked by GITRL present in the dermis.

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Induction of Cytokine (IL-1α, TNFα) and Chemokine (CCL20, CCL27, CXCL8) Alarm Signals after Allergen and Irritant Exposure

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Topical exposure to chemicals damages the skin and generates alarm signals which are produced by residential cells of the skin. The alarm signals call the immune system into action. We examined the nature of these alarm signals after exposure of skin residential cells to contact allergens and a contact irritant. Contact allergens (nickel sulphate and potassium dichromate) and irritant (SDS) were topically applied to the stratum corneum of human skin equivalents (fully differentiated epidermis on a fibroblast populated collagen gel) in a similar manner to that done *in vivo*.

All chemicals tested resulted in an identical time- and concentration dependent increase in pro-inflammatory cytokine (IL-1α and TNF-α) and chemokine (CCL20, CCL27 and CXCL8) secretion. Exposure to nickel sulphate and SDS were investigated in more detail: similar to chemokine secretion no difference was observed in the time- and concentration dependent increase in pro-inflammatory cytokine (IL-1α and TNF-α) secretion. After allergen or irritant exposure, neutralizing human antibodies to either IL-1α or TNF-α inhibited increased CCL20 and CXCL8 but not CCL27 secretion.

Our data shows that alarm signals consist of primary and secondary signals. IL-1α and TNF-α are released as primary alarm signals, which trigger the release of secondary chemokine (CCL20, CXCL8) alarm signals. However, some chemokines e.g.: CCL27 can be secreted in an IL-1α and TNF-α independent manner. We suggest that skin residential cells facilitate an allergic or an irritant contact dermatitis reaction by responding in a general manner to danger and in doing so initiate infiltration of immune responsive cells into the skin.

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Induction of TNF and β-Defensins in Murine Skin by *Staphylococcus aureus*

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The primary function of the skin is to form a barrier to prevent invasion of pathogenic microorganisms. Besides the physical barrier a chemical barrier involving β-defensins is important for host defence. β-defensins are cationic and cystein-rich antimicrobial peptides that control bacterial growth in many organisms and species by permealizing bacterial membranes. Recent studies have shown that murine β-defensin-1, -3 and -14 are homologues to the well-characterized human β-defensin-1, -2 and –3 most, which are important as defence against gram negative and positive bacteria. The functional conservation of the peptides allows experiments in a mouse model to develop new strategies for antimicrobial therapy. To investigate the induction of β-defensins, we applied soluble factors of the gram-positive bacterium *Staphylococcus aureus* on murine skin and determined at 1h, 3h, 6h, and 24h after treatment the mRNA expression of the proinflammatory cytokine TNF and the β-defensins m βD-1, -3 and –14. TNF-mRNA was significantly increased already 3h after *Staphylococcus aureus* application and still slightly enhanced 6h. β-defensin 1 was constitutively expressed in normal mouse skin; surprisingly an increase in the expression level occurred 6h after treatment. β-defensin-2 and -3 were not found in normal skin, however, induced 6h after treatment. We hypothesize host defence against *Staphylococcus aureus* leads to an early induction of TNF that in term may stimulate defensin expression.

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Regulation of Interleukin-20 Gene Expression in Normal Human Keratinocytes *in vitro*

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Interleukin-20 (IL-20) is a recently discovered member of the IL-10-family of cytokines. The skin of transgenic mice over expressing IL-20 show histological changes resembling some of those seen in psoriasis; i.e. thickened epidermis, hyperkeratosis and a compact stratum corneum. IL-20 mRNA has also been shown to be over expressed in psoriatic lesional skin compared to non-lesional skin, where it was barely detectable, indicating a role for IL-20 in the pathogenesis of psoriasis. At the same time keratinocytes were shown as the predominant cellular source for IL-20 mRNA expression in lesional psoriatic skin. However, the precise stimuli causing IL-20 gene expression in human keratinocytes has not yet been studied. The purpose of this study was therefore to identify various factors that can induce IL-20 gene expression in human keratinocytes, *in vitro*.

Cultured normal human keratinocytes were grown to approximately 70% confluency and then stimulated in separate experiments with a panel of different stimuli (IL-1α, IL-1β, UVB, bovine serum, IFN-γ, LPS, IL-6, IL-20, TNF-α, calcium, spontaneous differentiation) for 2 and 6 hours. Total RNA was purified from the cells and qRT-PCR analysis carried out in order to determine IL-20 mRNA-levels.

Stimulation with IL-1α, IL-1β, UVB and bovine serum lead to a pronounced and significant increase in IL-20 gene expression compared to control whereas IL-6 and TNF-α only gave rise to a small and insignificant increase in IL-20 gene expression. IFN-γ, LPS, calcium, IL-20 and spontaneous differentiation did not induce IL-20 gene expression. IL-1α (10 ng/ml) and IL-1β (10 ng/ml) were the most potent inducers of IL-20 mRNA expression and lead to a 5-fold increase compared to controls. IL-20 mRNA expression was maximal after 1 h of stimulation and reached base-line level after 6 hours. The response was dose-dependent with a maximal response 5ng/ml.

These results indicate that IL-20 gene expression in normal human keratinocytes is regulated through specific signal transduction pathways because IL-1α and IL-1β potently induced IL-20 mRNA expression whereas other inducers of inflammation such as TNF-α, LPS and IFN-γ were unable to induce IL-20 gene expression.

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Production of Human β-defensin-2 by Human Keratinocytes is Induced by Selective Strains of *Propionibacterium acnes*

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The Gram-positive, anaerobe bacteria *Propionibacterium acnes* (*P.acnes)* is found in normal human cutaneous flora and is thought to play major role in acne, a chronic inflammatory disease of the pilosebaceous unit. Although several lines of evidences suggest the direct role of *P.acnes* in the pathogenesis of acne, little is known about the mechanism by which *P.acnes* contributes in acne formation. In this study we investigated the capability of different strains ($n = 4$) of *P.acnes* to activate the innate immune response of the skin and the role of the antimicrobial peptide human β-defensin-2 (hBD2) as a possible regulator protein in *P.acnes* infection. We found that distinct strains of the bacteria generate different patterns of antimicrobial peptide and proinflammatory cytokine expression at both mRNA and protein level. Two of the clinically isolated strains significantly ($p < 0.05$) up-regulated the expression of hBD2, IL-8 and TNFα 3 hours after the treatment. In addition, *P.acnes* induced increase in hBD2, IL-8 and TNFα gene expression could be inhibited by anti-TLR2 and/or anti-TLR4 neutralizing antibodies. In conclusion our findings demonstrate that distinct clinical isolates of *P.acnes* up-regulate hBD2, IL-8 and TNFα expression in keratinocytes via the Toll like receptors (TLRs). This activity of *P.acnes* may be particularly important in the acute infection of the skin in which hBD2 participates in bacterial clearance. In addition, our findings stress the importance of hBD2 as the possible key regulator protein in *P.acnes* infection and acne formation.

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Characterization of Ovalbumin (OVA) Induced Atopic Dermatitis (AD) Murine Model

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Well-established AD murine model induced by OVA epicutaneous sensitization has been used world wide for studying the mechanisms underlying the disease. Little has been known about the progressing details of the model. The purpose of this study was to examine kinetically the development of AD at different stages in the AD murine model.

BALB/c mice were epicutaneously sensitized with OVA (OVA group) or saline (SAL group) for three weeks with a two-week-resting-interval between each sensitization week. Mice were sacrificed after each sensitization week. The last groups of mice were killed two weeks after the 3rd sensitization week. Cytokine and chemokine mRNA expressions in local skin tissue were investigated by real time PCR. Skin morphology was examined by histology methods. Total and specific antibody levels were studied by ELSA.

Compared with SAL groups, there were progressive and significant increases in both total and OVA specific IgE levels in OVA groups after the 2nd and 3rd sensitization week. The values dropped but kept significantly higher two weeks after the 3rd sensitization. Prominent skin inflammation was observed in OVA groups after the 1st sensitization week, which became most serious after the 3rd sensitization week. The skin recovered completely two weeks after the 3rd sensitization week. mRNA expressions of Th2 type cytokines, several chemokines and chemokine receptors were significantly higher in OVA groups with increasing tendency following each sensitization week. The levels fell to normal two weeks after the 3rd sensitization week.

In conclusion, skin inflammation and cytokine and chemokine mRNA expressions in OVA induced AD murine model started early after 1st sensitization week. IgE antibody response began after the 2nd sensitization week. The values were progressively increasing after each sensitization week and tended to become normal two weeks after the last sensitization week.

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Interlukin-1 alpha and beta are Potent Inhibitors of Connective Tissue Growth Factor Expression in Fibroblasts

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Background. Connective tissue growth factor (CTGF) is intimately involved with tissue repair and overexpressed in various fibrotic conditions. CTGF expression in fibroblasts is upregulated by transforming growth factor (TGF)-beta, and there is evidence suggesting that CTGF is mediating some of the profibrotic effects of TGF-beta. We have previously shown that keratinocytes *in vitro* downregulate basal and TGF-beta-induced expression of CTGF in fibroblasts by an interleukin (IL)-1alpha-dependent mechanism. Here, we investigated further the mechanisms of this downregulation and analysed if the IL-1 beta isoform also acts inhibitory on CTGF expression.

Methods. Human dermal fibroblasts and NIH 3T3 cells were treated with IL-1 alpha and beta in presence or absence of TGF-beta 1. CTGF mRNA and protein levels were measured by Northern and Western blotting, respectively. Activity of the CTGF promoter and TGF-beta-responsive promoter elements was determined by transient transfections of luciferase reporter constructs. Phosphorylated Smad 2/3 was analysed by immunoprecipitation and Western blotting.

Results. Both IL-1 isoforms suppressed basal and TGF-beta-induced CTGF mRNA and protein with a IC_{50} value of around 10 pg/ml. Pretreatment of cells with indomethacin did not abolish the effect, suggesting that IL-1-mediated CTGF suppression is not mediated via prostaglandins. CTGF promoter activity induced by TGF-beta was inhibited by IL-1alpha and beta. Likewise, TGF-beta-induced activity of a synthetic minimal promoter containing Smad binding CAGA elements was inhibited by IL-1 alpha and beta. Finally, TGF-beta-induced Smad 2/3 phosphorylation was inhibited by IL-1 alpha and beta.

Conclusion. IL-1 alpha and beta were found to be potent inhibitors of CTGF expression at the promoter, mRNA and protein levels. Our results support the view that IL-1 suppress TGF-beta-induced CTGF expression by inhibiting Smad signaling pathways without affecting prostaglandin production. These results add to the understanding of regulation of CTGF expression, which may aid in elucidating the pathogenesis of fibrosis.

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Adduct Formation Between Nucleophilic Amino Acids and Hexahydrophtalic Anhydride (HHPA), an Inducer of Both Type I and Type IV Allergy

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HHPA has a strong capacity to induce allergic asthma mediated by specific antibodies. It is also a well-known contact hapten giving rise to allergic contact dermatitis. Although adducts of HHPA to endogenous proteins have been described the epitope responsible for inducing allergy has not been established. In this study we chemically characterise the adducts formed *in vitro* between HHPA and nucleophilic amino acids and also towards a model peptide including all essential nucleophilic amino acids. The adducts were analysed and purified by HPLC and LS-MS. Their chemical structures were established by NMR and by nanospray hybrid quadropole-time- of-flight mass spectrometry. We found addition of HHPA to nitrogen as well as to sulphur giving major adducts to proline, lysine and cysteine. We also found that the originally formed HHPA-cysteine adduct reacted with lysine giving a thermodynamically more stable HHPA-lysine adduct. Studies on a model peptide with essentially all nucleophilic amino acids showed a dominant addition of HHPA to the N-terminal proline but at molecular excess of HHPA also addition to lysine and cysteine were registered. In this study we show how HHPA can react with different amino acid moieties present in proteins. This variety of reactivities gives rise to different epitopes. The knowledge is important in coming work on synthetic complete antigens for production of specific T-cells hybridomas. If addition to different types of amino acids or bonding atoms is essential for the type of allergic reaction or if the same complete antigen can induce several types of allergies is a question of comming research.

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Differential Expression of β-Defensins in Healthy Human Skin, Psoriasis and Eczema

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Defensins are a group of small, cationic, cysteine-rich peptides with broad-spectrum antimicrobial activity and were recently shown to represent an important part of the innate immunity of the skin organ. In this study the expression of human ß-defensins (hBD) 2 and 3 was examined in skin samples obtained from different body sites and inflammatory skin disorders. hBD-2 and hBD-3 mRNA was determined by *in situ* hybridisation using a specific probe generated in house on paraffin sections of healthy human skin of different localisations and in biopsies from patients with eczema, psoriasis and lichen ruber.

Expression of hBD-2 mRNA was found in only 50% of all samples tested, especially in areas which are highly exposed to both microbial challenge and UV-radiation (e.g. nose, forehead). In contrast, hBD-3 mRNA was determined in all skin samples tested. The expression patterns of both β-defensins were equal with a hybridisation signal most pronounced in the malpighian layer of the epidermis. In addition, in some samples sweat glands were also stained positively.

Expression of hBD-2 mRNA was detected in all lesions of lichen ruber, in 83% of eczematous skin but only in 40% of psoriatic skin. The intensity of the hybridisation signal was strongest in biopsies of psoriatic skin. All suprabasal layers were stained positively, whereas in samples from lichen ruber and eczema only the stratum granulosum showed staining for hBD-2 mRNA. No staining of endothelial or inflammatory cells was observed.

HBD-3 expression was found in all biopsies of psoriasis and lichen ruber but in only 66% of eczema lesions. The staining pattern was similar to that detected for hBD-2.

This study demonstrates the differential expression of hBD-2 and hBD-3 in healthy human skin. Partly absent expression could be explained by the fact, that hBD-2 and hBD-3 are inducible and not constitutively expressed antimicrobial peptides. Since hBD-3 is extremely active against Staphylococcus aureus, its lower expression in eczema lesions may explain the clinical experience, that atopic in contrast to psoriatic skin is prone to staphylococcus aureus infections.

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Human Keratinocytes Respond to IL-18. Implication for the Course of Chronic Inflammatory Skin Diseases

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IL-18, initially termed IFNγ-inducing-factor, has been described to decisively influence the course of several inflammatory skin diseases such as eczema and psoriasis. In this study, we aimed to further elucidate the keratinocytes role in the "IL-18" system. Here we show for the first time that human keratinocytes do respond to IL-18. In this study we were able to show that the expression of IL-18R alpha, responsible for IL-18 binding, is differentially expressed on keratinocytes. There is a clear basal expression of the receptor. A marked up regulation is induced upon stimulation with IFNγ plus a second signal such as TNF alpha. IL-4 was able to downregulate the IL-18R alpha expression. Keratinocytes showed activation of the NF-kB signalling pathway upon IL-18 stimulation as detected by nuclear translocation. The main biological effect of IL-18 was induction of MHC class I and II expression on human keratinocytes, known as non-professional antigen presenting cells. The MHC II expression was of functional significance as determined by stimulation of autologous CD4 + T-cells in the presence of superantigen: CD4 + T cells cultured with MHC II expressing keratinocytes induced by IL-18 and stimulated with SEB produced significantly higher amounts of IFNγ as compared to T cells from control cultures. In freshly isolated skin biopsies from patients with epidermal inflammatory skin diseases we observed a markedly higher expression of the IL-18R alpha on keratinocytes from atopic dermatitis and psoriasis patients as compared to normal donors. In conclusion, we have shown evidence that keratinocytes – as the outermost component of the skin – functionally respond to IL-18. The receptor is upregulated in inflammatory skin diseases pointing to the fact that IL-18 could decisevely contribute to chronification of skin diseases via the keratinocytes response.

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Tributyltin (TBT) Induces an Exacerbation of Atopic Dermatitis

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Background. Tributyltin (TBT) has been used for antifoulants on boats, ships, quays, buoys, crab pots, fishnets, and cages. TBT has been well known as an endocrine disrupture. It is reported that it affects on human immune system. However, it is not clear how TBT affect on human immune system. Recently, atopic dermatitis (AD) has been increasing in the developed countries. It seems that endocrine disruptures, such as TBT, influence the ratio of prevalence. TBT may have an influence on immune system, and cause atopic allergic disease such as AD.

Objective. We investigated how TBT affects on Th1/Th2 balance in murine immune system and on AD-like eruption in murine model of AD.

Method: We demonstrated DS-Nh mice, which spontaneously develop dermatitis similar to human AD under conventional condition. After they were fed with or without TBT diet for 30 days, they received topical TNCB treatment on the dorsal skin surface. We examined the skin eruption score, histology of skin with H-E staining, serum total IgE levels, and cytokine production by splenocytes and lymphnode cells stimulated with SEB or ConA by ELISA. We also counted the number of colonies of S.aureus on the skin surface, and examined T cell subsets by FACS analysis.

Result & Conclusion. Mice treated with TBT showed severe AD-like skin eruption with increased number of colonies of S.aureus in comparison with non-treated mice. However, serum levels of total IgE of TBT treated group were decreased rather than increased compared with that of control group. The mice exposed to TBT showed also decreased amounts of IFN-γ production by splenocyte or LN cells. The proportion of CD25 + CD4 + T cells and NK cells were significantly decreased in TBT treated mice compared with control mice. In contrast, only IL-13 production by splenocytes or LN cells from TBT treated mice were significantly increased compared with that from control mice. Mice exposed to TBT for 30 days showed increased Th2 response and reduction in number of regulatory T cells (CD25 + CD4 + T cells). These results indicate that TBT may contribute exacerbation of AD-like eruption.

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Adherent, Mucoid *Pseudomonas aeruginosa* Release Pathogen-associated Molecules Inducing Macrophage Inflammatory Protein-3α in Keratinocytes

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Keratinocytes represent the first cellular line in the host defense against microorganisms like *Pseudomonas aeruginosa* (PA). They are capable of producing antimicrobial agents like defensins and releasing leukocyte chemoattractants to fight microbial infection. However, little is known about the molecular interaction of pathogen associated molecules (PAMs) with keratinocytes leading to immune responses. We hypothesize that Macrophage Inflammatory Protein-3α (MIP-3α) released by keratinocytes upon contact with microorganisms, may play an important role in initiating adaptive immune responses by recruiting immature dendritic cells. We investigated whether bacteria release compound PAMs upon contact with skin, which induce MIP-3α in keratinocytes. Initial experiments revealed indeed MIP-3α induction by bacterial PAMs.

To investigate under which conditions bacteria release these PAMs the effect of bacterial culture conditions on the production of MIP-3α-inducing PAMs was analysed. Supernatants of PA cultured under unstressed or stress conditions (low oxygen, starving conditions) were used to investigate the effects on MIP-3α-induction in HaCaT keratinocytes. RNA was isolated and the relative MIP-3α mRNA expression was determined using gene-specific primers in Realtime-PCR.

We found that supernatants of PA cultivated under stress conditions, which led to adherent, mucoid phenotypes, induced expression of MIP-3α, whereas supernatants of unstressed cultures lacked such MIP-3α-inducing PAMs.

Initial experiments to characterize MIP-3α-inducing PAMs by size exclusion chromatography revealed that these PAMs seem to be heterogeneous showing a molecular mass between 200–600 kDa. Moreover, they seem to be different from PAMs inducing antimicrobial peptides which elute from the column in the effluent corresponding to masses >600 kDa. Anion exchange chromatography revealed that MIP-3α-inducing PAMs have a slight anionic charge.

Our investigations confirm that human skin keratinocytes recognise bacterial PAMs and mount an innate defense by production of chemokines like MIP-3α and antimicrobial peptides. Our results indicate that solely stressed adherent bacteria release such MIP-3α-inducing PAMs. These bacterial PAMs are different from those inducing antimicrobial peptides and proteins in keratinocytes.

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Pseudomonas Aeruginosa Represses Interleukin-1 Mediated Inflammatory Response and Beta-Defensin-1 and -4 in Murine Epidermal Cells

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Pseudomonas aeruginosa (PA) infections result in delayed wound healing or compromise immunosupressive conditions, despite immune responses from the host organism. The impacts of PA on keratinocyte gene regulation, signal transduction and immunity are not yet established. We investigated changes in gene profiling after PA stimulation by treating murine PAM212 keratinocyte cell line with PA culture supernatant and analysing gene expression with a customised micro array technique. 24 hours after PA treatment, we extracted total RNA through reverse transcription and generated *in vitro* cRNA probes. Fragmented cRNA was hybridised to the micro array containing oligonucleotide probe sets representing more than 400 transcripts. The hybridised micro arrays were scanned and genes were filtered, producing signals stronger than an arbitrary cut-off point for florescent signal intensity (more than 1.4 fold increase or decrease and consistent expression of selected genes in three analyses). After PA stimulation we found higher expression of proliferation associated mRNA of angiotensin-2 receptor and small proline-rich protein-1, and down regulation of interleukin-1 receptor-1 family signal molecules (inner ligands myd88, irak-1, and traf-6). The expression of genes of the innate immunity system were also affected by PA stimulation: beta-defensin-1 and -4 and lipopolysaccharide binding protein were down-regulated. Expression of differentiation markers filaggrin, desmoglein-1 and –3, envoplakin, periplakin, and transglutaminase processing cathepsin D was reduced. In summary, PA represses interleukin-1 mediated inflammatory response, beta-defensin-1 and -4, and cell differentiation. We suggest that PA infection may persist due to down-regulation of the host immune response targeted against the bacteria.

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Cytokines Expression of Human Epidermal Langerhans cells: An Update

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Due to the difficulties in isolating pure preparations of epidermal Langerhans cells (LCs), their production of inflammatory cytokines is still far from clear. We have recently established a protocol for isolating and culturing ultrapure and viable human LCs with the morphologic, phenotypic and functional characteristics of epidermis-resident immature cells. Our Multiplex-PCR analyses demonstrated that unstimulated LCs are characterized by constitutive expression of IL-6, IL-8 and TGF-β. Stimulation with LPS induced transcription of IL-1β, TNF-α and of GM-CSF, with transcripts for TGF-β and GM-CSF in 99% of the CD1a-positive LCs. Data from gene array experiments revealed differential gene expression in LCs that were stimulated by a combination of CD40 ligand and LPS for 4 h. Most interestingly, there was no significant transcription of IL-12, but we found expression of the newly described proinflammatory cytokine IL-27. After stimulation, the gene of the subunit p28 was expressed by LCs and not by stimulated keratinocytes from the same donor. The LCs showed in addition transcripts of IL-15 and IL-18. In conclusion, resting and stimulated human epidermal LCs are characterized by distinct cytokine profiles, different from their *in vitro*-generated counterparts. It will be of interest to determine the contribution of each of these cytokines in innate and adaptive immune responses, and in particular, to study the role of IL-27 in Th1 development.

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The pro-inflammatory Cytokines, IL-1α and IFN-γ Induce Distinct Transcriptome Signatures in Human Keratinocytes

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As the predominant cell type within the epidermis, keratinocytes have been demonstrated to be pivotal in the initiation maintenance and regulation of immune responses in the skin. We were interested to determine the effect of IL-1α and IFN-γ, two key cytokines relevant to inflammatory skin disease, on the transcriptome of keratinocytes using Affymetrix GeneChip technology. Both IL-1α and IFN-γ are potent, pro-inflammatory and pleiotropic, but differ in their association with the two major types of immune response; innate (IL-1α) and adaptive (IFN-γ). Therefore, in addition to acquiring comprehensive information about the transcriptional response to these cytokines in keratinocytes, the study attempted to gain more general insights into the two types of immune response in epidermis.

Second passage human keratinocytes, derived from normal adult donors undergoing breast reduction surgery, were stimulated with either IL-1α (100 ng/ml) or IFN-γ (20 ng/ml) for 24 hours prior to isolation of total RNA, first and second strand cDNA synthesis and preparation of biotinylated cRNA (n = 3). Samples were subsequently hybridized to Affymetrix U133A human microarrays (containing oligonucleotides representing approximately 22,000 transcripts), scanned and analysed using dChip software to identify transcripts showing a consistent difference of at least 2-fold between any 2 arrays. Stimulation with IFN-γ resulted in consistent strong induction (> 5 fold) of a number of the CXCR3-binding ligands, CXCL9, CXCL10 and CXCL11 and class II MHC genes, particularly those of the HLA DR family. By contrast, the keratinocyte transcriptome resulting from IL-1α stimulation exhibited strong induction of a number of proteins in the epidermal differentiation complex (EDC) (eg. S100 A7/psoriasin), protease inhibitors (eg. SCCA1), antimicrobials including SKALP/Elafin and β-defensin 2 and the chemokines IL-8 and CCL20. Further, direct comparisons between arrays hybridised with the two cytokines revealed high specificity of the responses with markedly different (at least 5-fold) expression of groups of transcripts representing T-cell chemokines and MHC/antigen presentation molecules following IFN-γ stimulation, compared with EDC molecules, antimicrobials and markers of abnormal differentiation following IL-1α stimulation.

These findings demonstrate a marked difference in the transcriptome of keratinocytes stimulated with two functionally distinct cytokines and suggest that innate and adaptive immune responses within the epidermis involve the coordinated expression of groups of related proteins with discrete functions. These findings are of added interest when compared with previous microarray studies we have performed using psoriatic epidermal shave biopsies, in which the lesional transcriptome displayed highly significant overlap with the profiles seen following IL-1α stimulation in the present study, suggesting that the inflammatory milieu in the epidermal microenvironment in psoriasis is more dependent on an innate, rather than the expected adaptive response.

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Allelic Variation in the TGF-beta1 Gene T869C Polymorphism Does Not Explain Fibroblast Activation And Collagen Synthesis in Systemic Scleroderma and Morphea Patients

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Systemic scleroderma (SSc) or progressive systemic sclerosis occurs sporadically, except isolated cases with family history, suggesting a multifactorial genetic predisposition. Ohtsuka *et al*. reported an significant association of the rare C-allele of the 869 bp T to C transition at amino acid position 10 (Leu to Pro) of the human transforming growth factor (TGF)-beta1 gene in 59 patients with SSc (Br J Dermatol 147:458, 2002). TGF-beta has been formerly identified as potent promoter of fibroblasts and collagen synthesis and is furthermore involved in the pathogenesis of sclerosis of the skin and the lung tissue in SSc. To further investigate the TGF-beta1 polymorphism in position 869 bp, we have isolated genomic DNA from 50 patients with diffuse SSc (dSSc), 40 patients with limited SSc (lSSc), 13 morphea patients and 53 healthy control individuals. A 288 bp fragment carrying a polymorphous MspAII restriction site was amplified by standard PCR techniques and digested by the enzyme. The C-allele was characterized by restriction of the amplificate and typed by gel electrophoresis and DNA sequencing. The distribution of the rare C-allele in dSSc (C/C 8%, C/T 54%) did not differ significantly from lSSc (C/C 10%, C/T 52.5%) and from the controls (C/C 19%, C/T 49%). If regarded at severity and clinical features typical for SSc, like sclerodactyly (N = 77), lung involvement (N = 39), kidney involvement (N = 31) and autoantibodies against topoisomerase I (Scl70, N = 33), again, no significant association of the T869C polymorphism could be detected. The results presented here argue against a particular involvement of the T869C polymorphism of the TGF-beta1 gene in the pathogenesis of perturbed collagen synthesis in our sample of Caucasian patients with SSc and morphea, although we investigated only a very limited number of morphea patients.

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Evaluation of the Potential Role of Cytokines in Toxic Epidermal Necrolysis

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Toxic epidermal necrolysis (TEN) results from the widespread apoptosis of epidermal cells. The mechanisms leading to this apoptosis are not yet elucidated.

We investigated whether the cytokines present in the blister fluid which accumulates under necrotic epidermis originated from T-lymphocytes and may play a role in the propagation of keratinocyte apoptosis. IFN-gamma was elevated in 12 cases, sTNF-alpha in 9, sFas-L in 9. High concentrations of IL-10 were found in all 10 patients tested. In the control blister fluids from 3 burned patients we found high concentrations of IL-18, slight elevations of IL 10 in 2 cases, of sTNF-alpha in one and normal levels of IFN-gamma and sFas-L.RT-PCR studies of mononuclear cells present in the fluid of TEN patients indicated that only IFN-gamma and to a lesser extent IL-18 were produced by these cells. That suggested that other cytokines also present (TNF-alpha, sFas-L, IL-10) rather originated from activated keratinocytes. Fas-L was actually over-expressed on the membrane of keratinocytes in lesional skin *in situ* as already reported by others indicating that keratinocytes were the source of sFas-L in the blister fluid. The expression of Fas in lesional skin was mainly limited to basal cells.

The Th1 profile of T-lymphocyte activation found in the blister fluid of patients with TEN is consistent with a key role for the drug specific cytotoxic lymphocytes that we previously found in the blisters. The CTL were only lytic on keratinocytes activated by IFN-gamma. Blister fluid contained enough IFN-gamma to activate keratinocytes but did not incuce apoptosis in the absence of CTL. We propose the hypothesis that the production of Fas-L, TNF-alpha and IL-10 by keratinocytes is more a defense mechanism against cytotoxic lymphocytes than a way of propagating apoptosis among epidermal cells.

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Expression of IL-23 by Keratinocytes in Healthy Skin and Psoriasis Lesions

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Keratinocytes are known to contribute to the cutaneous immune responses by interacting with immunocompetent cells and expression of cytokines. In addition to proinflammatory cytokines, they were found to express type 1 cytokine, i.e. interferon (IFN)-γ - inducing cytokines, such as interleukine (IL-12) which is a heterodimer formed by p40 and p35 subunits. The newly defined IFN-γ inducing cytokines IL-23 and IL-12 share the p40 subunit in their heterodimeric structure. We investigated whether keratinocytes can express the p19 subunit of IL-23 and the biologically active heterodimer IL-23.

Skin biopsies from normal individuals (n = 13) and psoriatic patients (n = 16) were used in this study. Keratinocyte cultures from normal, nonlesional and lesional psoriatic skin were used to determine the expression of IL-23 at mRNA level using RT-PCR technique. Frozen sections were used to perform immunostaining with antibodies against p19 and p40 subunits of IL-23. Freshly isolated and cultured keratinocytes were immunostained with the same antibodies as well. Supernatants and cell lysates from stimulated keratinocytes were used to determine the expression of IL-23 by immunoblotting.

Both p40 and p19 subunits of IL-23 were expressed at mRNA and protein level in freshly isolated and cultured keratinocytes as determined by PCR and immunostaining, respectively. In situ, these subunits were expressed at a higher level in psoriatic lesional keratinocytes than in nonlesional and normal keratinocytes. By immunoblotting, the active heterodimer of IL-23 was determined in supernatants and cell lysates of *in vitro* stimulated keratinocytes.

These findings demonstrate that keratinocytes are capable of producing p19 subunit and the biologically active form of IL-23. Psoriasis is a chronic inflammatory skin disease mediated by IFN-γ expressing type 1 memory T cells. As IL-23 is important to activate memory T cells to produce IFN-γ, its augmented expression by keratinocytes can contribute to the inflammatory changes which are observed in psoriatic skin.

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Lipid Oxidation Products Induce Expression of the Anti Inflammatory Protein Heme Oxygenase –1 in Keratinocytes, Dermal Fibroblasts and Epidermal Equivalent

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A specific class of phospholipid oxidation products i.e. oxidized L-alpha-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholin (OxPAPC) that have been found in cell membranes of apoptotic cells, oxidized low-density lipoproteins, and at sites of inflammation, have recently been shown to directly act as potent inhibitors of acute, LPS-dependent inflammation. In addition, it was demonstrated that OxPAPC induces expression of Heme Oxygenase-1 (HO-1), an enzyme with potent anti-inflammatory properties, adding an unrelated mechanism by which oxidized phospholipids may influence inflammatory reactions.

When we studied the effects of lipid oxidation products on primary KC, dermal fibroblasts, HACAT and in an epidermal equivalent model, we found that OxPAPC induced expression of both HO-1 mRNA and protein. Addition of the radical scavanger bHT (butylated hydroxytoluene) did not inhibit the induction of HO-1 expression, indicating this process was not dependent on putative oxidative properties of OxPAPC. Since HO-1 has been implicated to play a role in wound healing by degrading free heme and thereby preventing free radical formation and lipid peroxidation, our data suggest that its induction by oxidized phospholipids represents a potential new therapeutic approach in the treatment of skin diseases associated with tissue damage mediated by oxidative stress.

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Influence of Chronic UVB Exposure on the Proinflammatory Cytokines' Profile Alteration

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Ultraviolet irradiation exposure causes inflammatory changes in the skin and an increase in expression of proinflammatory cytokines. The aim of the study was to assess serum level of proinflammatory cytokines (IL-1, IL-6, IL-8, TNF-alpha) after a 10-day exposure to suberythemal dose of UVB and subsequent irradiation with an acute UVB dose. The study included 30 healthy volunteers aged 18–53years old. Individual MED was assessed (mean 0.15 J/cm^2). Later blood samples were taken from each subject and a 10-day exposure to 0,7 MED irradiation (whole body) started. 24 hours after a final irradiation blood sample was taken and subsequently local irradiation (buttock, 3 MED) was applied. Next day third blood sample was taken. Serum concentration of cytokines was determined by chemiluminescence assay and the results were statistically analyzed. A statistically significant increase in serum concentration of IL-8 ($p<0{,}005$) and a tendency of increased TNF-alpha ($p>0.05$) concentration were found. Serum concentration of IL-8 and TNF-alpha after 3 MED irradiation decreased to the base levels. No changes in IL-1 and IL-6 serum level were found. Conclusion: Chronic suberythemal doses of UVB irradiation enhance IL-8 serum concentration and they are likely to make adaptation in regard with biological effects caused by acute irradiation.

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Long-term Treatment with Etanercept Significantly Reduces the Number of Proinflammatory Cytokine-Secreting Peripheral Blood Mononuclear Cells

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Objective. Etanercept is effective in the treatment of chronic inflammatory diseases. The present study analyzed the influence of etanercept treatment on the number of peripheral blood mononuclear cells (PBMC) secreting immunoregulatory key cytokines in patients with rheumatoid arthritis (RA). The cell counts were correlated to the therapy response.
Methods. Nineteen patients with longstanding, active RA were treated with etanercept as monotherapy. Frequencies of PBMC secreting tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10 and interferon (IFN)-γ were determined by ELISPOT analysis before and after 9 months of therapy and compared to healthy controls (HC). The clinical outcome was assessed as defined by the American College of Rheumatology criteria.
Results. The clinical response to etanercept was excellent, after 9 months of therapy 15 patients fulfilled the ACR20, 7 patients the ACR50 and 2 patients the ACR70 criteria. Initially elevated numbers of TNF-α and IL-1β secreting PBMC were reduced to HC levels, normal numbers of IL-6 and low numbers of IFN-γ secreting PBMC to below HC levels. The number of IL-10 secreting PBMC did not differ from that in HC and did not change significantly over time. The pretreatment IFN-γ : IL-10 ratio correlated to reduction in the tender ($r=0.48$; $P<0.05$) and swollen ($r=0.51$; $P<0.05$) joint count within 9 months.
Conclusion. Long-term treatment with etanercept significantly reduces the number of proinflammatory cytokine secreting PBMC, while the number of IL-10 secreting cells is unaffected. Although these immunologic alterations did not affect the safety or efficacy of etanercept therapy, they may account for the long-term systemic effects. The pretreatment IFN-γ : IL-10 ratio may be of prognostic value in predicting the improvement in joint symptoms.

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TNF-α, IL10, IL6 and IL1RA Gene Polymorphism in Leprosy in an Egyptian locality

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The variability of the host response to M.leprae infection seems to be genetically and environmentally influenced. Many host genes are involved in a complex interplay of host gene regulation that leads to disease. Cytokines evidently play a critical role in triggering host–pathogen interaction. The aim of this work was to investigate the presence of certain cytokine gene polymorphisms that may be important in the pathogenesis of leprosy. This study included 47 leprosy patients and 42 healthy controls. By DNA extraction and PCR amplification gene polymorphism for TNF-308; IL10-1064/-1082; IL6-174; IL1RA-VNTR were studied. Although the combined genotype frequencies of all studied cytokine polymorphisms showed that certain genotype {IL10 (1,2); IL-6(1,2); TNF(1,2); IL1RA(2,2)} was the most common among the studied subjects being higher in controls than cases, the frequency of the genotype {IL10(1,1);IL6 (1,2);TNF(1,2);IL1RA(2,2)} was higher in cases than controls. IL10 allele 1 and IL6 allele 2, TNF allele 2 and IL1RA allele 2 were more common among cases compared to controls. A significant difference was detected between cases carrying those alleles and others carrying the other alleles ($P=0.002$) with variations regarding different clinical types of leprosy. In conclusion, certain combined genotype frequencies may determine susceptibility to develop clinical presentation of leprosy.

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Increased Expression of the Novel Proinflammatory Cytokine HMGB1 is Detected in Cutaneous Lupus Erythematosus Lesions

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The aim of this study was to investigate the expression of proinflammatory cytokines in lesions and unaffected skin of patients with cutaneous lupus erythematosus (CLE), to identify factors of immunoregulatory importance in disease manifestations.

Cutaneous lupus erythematosus is an autoimmune disease associated with the presence of Ro/SSA autoantibodies. Subacute cutaneous lupus erythematosus (SCLE) and chronic cutaneous lupus erythematosus (CCLE) are subsets of CLE. Discoid LE (DLE) is the most common form of CCLE. In the affected skin of these conditions mononuclear cell infiltrates develop. The infiltrates consist mainly of T cells, which may be activated by expression of proinflammatory cytokines. The novel cytokine HMGB1 (high mobility group box chromosomal protein 1) is an intranuclear protein. One of its cellular functions is in organization of chromatin and structuring of DNA. During inflammation, it is translocated to the cytoplasm in macrophages and released extracellularily, to act as a proinflammatory cytokine. Secretion of HMGB1 is induced by TNF and IL-1beta. TNF is an early mediator of inflammation, usually released within minutes of stimulation, and can induce the release of both HMGB1 and IL-1beta. IL-1beta also has broad proinflammatory activity. Like other proinflammatory cytokines, HMGB1 can activate monocytes which in turn stimulate the release of TNF and IL-1beta, promoting a loop of immune activation. To investigate factors of importance in activation of T cells within the skin lesions, we studied the expression of proinflammatory cytokines.

Skin biopsy samples from affected and unaffected (buttock) skin of 10 patients with CLE were investigated, of whom 6 had SCLE and 4 had DLE. Expression of HMGB1, TNF and IL-1beta was analyzed by immunohistochemical technique of cryosections.

As a ubiquitous nuclear protein, HMGB1 was expressed in both affected and unaffected skin specimens. In unaffected skin, HMGB1 was expressed in the epidermis as well as in and around vessels in the dermis. In affected skin, consistently higher degree of expression was observed intra- and extracellularily. The expression was predominately localized in and around the mononuclear infiltrates in the dermis. TNF was detected almost exclusively in affected skin specimen, and was predominately expressed in and around dermal mononuclear infiltrates. IL-1beta was expressed in both affected and unaffected skin specimens. In unaffected skin IL-1beta was expressed in the epidermis. In affected skin, IL-1beta was detected mostly in and around mononuclear infiltrates in the dermis, but also to a lesser degree in the epidermis. Analysis of consecutive sections demonstrated that IL-1beta and HMGB1 are widely expressed and in the same areas of the infiltrates, while TNF expression was restricted to subdomains of the infiltrates.

Our results suggest that HMGB1, together with TNF and IL-1beta, are of importance in the pathogenesis of cutaneous lupus erythematosus and constitute potential therapeutic targets.

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Release of Interferon-Gamma (IFNγ) to Multiple Drugs in Patients with Cutaneous Adverse Drug Reactions (CADRs) – A Possible Manifestation of Multiple Drug Allergy

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Multiple drug allergy syndrome is a clinical condition characterized by adverse reactions against more than one different class of pharmacologically and structurally unrelated drugs. The pathogenesis of multiple drug allergy is still unclear, although scanty data implied the existence of delayed-type hypersensitivity (DTH).

The aim of the study was to evaluate the clinical relevance of drug-induced *in vitro* interferon-gamma (IFNγ) release to multiple drugs in patients with cutaneous adverse drug reaction (CADRs).

The study comprised of a selected group of 15 patients with a variety of CADRs following intake of drugs, in whom positive IFNg release tests for 2 or more drugs (per patient) were obtained following incubation of peripheral blood lymphocytes with drugs. IFNg release was detected in the supernatants using a commercial ELISA kit. Twenty two patients taking a similar profile of drugs without developing an adverse reaction, served as controls. For each drug tested the increase in IFNg release was calculated. A value higher than the mean percentage increase of IFNg + 2SD measured in controls was determined as a positive IFNg test. Drugs taken by the patients were classified into 3 categories of clinical drug suspicion: high, possible and low. *In vivo* tests (withdrawal and/or challenge) with drugs were evaluated.

The main types of eruptions observed were vasculitis, maculo-papular eruption and urticaria. IFNg release for 58 drugs taken by the patients (101.6 $\pm$ 100.8%) was higher ($p<0.001$) than that recorded for 54 drugs taken by the controls (22.8 $\pm$ 25.9%). Positive IFNg release tests were recorded towards 65.5% of the drugs, including mainly NSAIDs, antimicrobials and analgesics. Positive IFNg tests were recorded for two drugs (67%), three drugs (13%) and four drugs (20%). The percentage of positive IFNg responses was directly related to the degree of clinical drug suspicion: 81.8%, 50%, and 42.1%, respectively. The percentage of positive IFNg responses recorded for the high-suspicion drugs was higher ($P=0.0065$) than that recorded for the two other drug categories. Percent agreement between the results of *in vitro* IFNg release tests and *in vivo* tests was 81.0%, kappa = 0.57, which implies intermediate to good agreement.

Clinical relevance was attributed to *in vitro* release of IFNg to multiple drugs in patients with CADRs. The study may further support the role of DTH and cell mediated immunity in the pathogenesis of multiple drug allergy.

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Platelet-activating Factor Enhances Motile Activity of Immature B Lymphoblastoid Cells

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Platelet-activating factor (PAF) is a biologically active ether phospholipid involved in inflammatory and immune responses. PAF has been reported to elicit infiltration of inflammatory cells into local environment via diverse pathophysiological actions including chemotaxis of neutrophils, eosinophils and dendritic cells. However, PAF effect as a chemoattractant for B lineage cells has not fully been explored despite of the fact that PAF receptor (PAF-R) was evidenced in a part of peripheral blood B cells, tonsillar B cells, B lymphoma cells and EB virus-transformed B lymphoblastoid cells (BLCs). In this study, we examined PAF-R expression in immature and mature BLCs and the chemotactic effect of PAF on BLCs. Flow cytometry analysis showed that PAF-R expression level was much higher in immature BLCs than mature BLCs. Additionally, immature and mature BLC subsets were examined for the number of functional PAF-R capable of binding to PAF by Scatchard analysis. Specific [^{3}H]PAF binding to immature subset was apparently higher compared with that to mature subset, implying the feasibility of the higher susceptibility of immature BLC subset to PAF effect. Chemotaxis analysis using modified Boyden chamber showed that PAF enhanced a motile activity of immature BLCs but not that of mature BLCs. These results suggest that PAF may serve as a chemotactic factor predominantly for immature B lineage cells rather than mature B lineage cells including antibody-releasing B cells, leading to relative increase of immature B lineage cells in inflammatory response or in B cell lymphoma invasion.

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Increased Interleukin-12 Expression in Vitiligo

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Interleukin 12 (IL-12) induces not only the production of type 1 cytokines like interferon-γ, but it also promotes cellular immune responses. In vitiligo patients melanocyte-specific autoantibodies as well as cytotoxic T cells have been found in the peripheral blood. In the present study we investigate the expression of IL-12 in vitiligo. Using real time quantitative reverse transcriptase polymerase chain reaction and immunohistochemistry we examined IL-12 in healthy controls and in lesional/perilesional and non-lesional skin of patients with vitiligo. The number of positive cells with monoclonal mice anti-hIL-12 p35/p70, hIL-12 p40/p70 and hIL-12 p70 antibodies was significantly increased in vitiligo lesions compared to control skin. IL-12 immunoreactivity was mainly located in the cytoplasm of dermal dendritic cells (DCs) and macrophages as well as in some epidermal Langerhans cells (LCs).

In conclusion our data suggest that the enhanced local production of IL-12 in DCs and macrophages may be responsible for the activation of cytotoxic T cells in vitiligo. They support the hypothesis that IL-12 may have a pivotal role in promoting melanocyte destruction in vitiligo by cytotoxic immune responses.

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Microheterogeneity of Selected Acute Phase Proteins in Patients Suffering From Mycosis Fungoides

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Mycosis fungoides (MF) is the most common form of cutaneous T-cell lymphomas (CTCL). The disease is characterised by long term clinical progression from patch through plaque to tumour stage. Erythrodermic variants also exist. Cytokine balance is considered to be of major importance for pathogenesis of MF. The aim of the study was to evaluate glycosylation profiles of selected acute phase proteins (APP) reflect the cellular processes in various stages of MF. Acute phase proteins may serve as a laboratory marker of cytokine network function: changes in APP level reflect changes in cytokine balance and are easier to follow analytically.

Sera samples were obtained from 52 patients with MF, aged from 33 to 82 years. Patients were classified after the staging evaluation into four groups: 13 patients with MF in patch stage (IA + IB), 12 patients with plaque stage (IIA), 12 patients with tumour stage (IIB), 15 patients with erythroderma (IVA). Sera from 20 healthy subjects served as controls. Concentrations of $alpha_1$-acid glycoprotein (AGP) and $alpha_1$-antichymotrypsin (ACT were measured using Laurell rocket electrophoresis, whereas glycosylation profiles were estimated using crossed affinity immunoelectrophoresis with Con A as ligand, acc. to Bog-Hansen. Results were analyzed using STATISTICA 6.0 Software.

An inflammatory reaction clearly exists in all MF patients, as the concentrations of AGP and ACT were increased. The lowest intensity of acute phase reaction is shown in plaque stage (IIA) of MF. An increase in the concentration of AGP was described previously in many inflammatory processes. Its synthesis is stimulated mainly by IL-6, suggesting a constant production of this cytokine in all except the plaque stages of MF. ACT concentration is related to necrotic processes or traumatic events with massive tissue damage. In patients with MF the concentrations of AGP and ACT seem to increase in parallel, as if stimulated by the same factor. Our results suggest that in a course of MF a generalised inflammatory reaction, known as acute phase response, is present in all patients. The changes of glycosylation profiles were seen in all except patients with patch stage MF, with the highest intensity for patients with erythroderma. It suggests that in early stage of MF the inflammatory reaction seems to be acute and changes during progression of the disease towards chronic image.

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Imiquimod Increased IL-6 Secretion from Normal Human Mixed Epidermal Cells

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Key words: imiquimod, IL-6, epidermal cells

In order to better understand the immunoregulatory mechanism by imiquimod, one kind of drugs widely used in the treatment of condyloma acuminatum (CA), we investigated the effects of imiquimod on IL-6 secretion from normal human mixed epidermal cells under culture condition. Single cell suspensions of normal human epidermal cells were prepared utilizing standard techniques, then cells were cultured with culture medium alone or with the medium containing 5μg/ml of imiquimod for 1, 2, 4, 8 and 12 hours. The cell-free and the conditioned medium supernatants were harvested and IL-6 secretion in the supernatants was assayed by using commercially available ELISA kits. We found that a low level of IL-6 secreted from mixed epidermal cells treated with the simple culture medium, but when incubated with imiquimod, the mixed epidermal cells could secrete a large amount of IL-6. At any time intervals, IL-6 secreted from imiquimod treated groups was higher than that from control cells ($p < 0.01$). IL-6 secretion reached its peak (245.1pg/ml) 8 h after imiquimod treatment compared with untreated group (97.2pg/ml). The results demonstrated that imiquimod can enhance the secretion of IL-6 from normal human mixed epidermal cells. Our data partially explain the immune regulatory mechanism of imiquimod.

252 [Oral 010]

The Balance between Nuclear Factor-kappaB and c-Jun N-terminal Protein Kinase/AP-1 Activity Controls Dendritic Cell Life and Death

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The duration of the lifespan of dendritic cells (DCs) must be tightly controlled for proper function of adaptive immunity. Some signals leading to enhanced DC longevity have been defined. Signaling pathways actively mediating DC death remained largely enigmatic. To elucidate how decisions on the fate DCs are made we aimed at identifying relevant transcriptional activators that positively or negatively regulate DC survival. We show in $CD34^+$ human progenitor cell derived DCs by means of reporter gene assays, that ligation of TNF-R superfamily (TNFR-SF) members on DCs (CD40, CD95, TRAIL-R) as well as cognate DC-T cell contact resulted in simultaneous and quantitatively balanced NF-kappaB and c-Jun N-terminal kinase (JNK)-mediated AP-1 induction and strongly enhanced DC longevity. When NF-kappaB activation was experimentally blocked by endogenous inhibitors (i.e. transfection with IkappaBalpha(S32A/S36A), JNK and, consequently, AP-1 activity was drastically augmented and TNFR-SF triggering and cognate T cell encounter resulted in DC apoptosis. Inhibition of the JNK/AP-1 pathway by pharmacologic compounds or dominant-negative JNK mutants rescued DCs from TNFR-SF member- and T cell-mediated death. We conclude that JNK/AP-1 activity is subject to negative feedback inhibition by NF-kappaB and can execute apoptosis in DCs if NF-kappaB activity is impaired. Thus, feedback-controlled signaling amplitudes of two transcriptional pathways decide upon the fate of a DC.

253 [Oral 028]

Immature, Semi-Mature and Fully-Mature Dendritic Cells Present in the Skin-Draining Lymph Nodes

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Dendritic cells (DCs) are characterized by their migration and maturation capacity. It is widely believed that immature DCs are distributed in all organs and mature ones appear in the secondary lymphoid organs. Here we show that in the steady state, there are phenotypically immature and semi-mature DCs in the skin-draining lymph nodes (LNs), while in the spleen only immature DCs are present. These semi-mature DCs are clearly distinguished from immature ones by high expression levels of MHC class II, B7-2, CD40 and CCR7. Any of myeloid, lymphoid and plasmacytoid DCs are included in both populations. The semi-mature DCs are completely absent in CCR7−/− mice and subcutaneous injection of pertusis toxin decreased this population in the draining LN, indicating that they are migratory cells mostly derived from the skin. In contrast, these semi-mature DCs are intact in TNFR1&2−/−, MyD88−/−, CD154−/− and germ-free mice, indicating that inflammatory stimuli, such as TNFα, IL-1, CD40 ligation and bacterial colonization, are not responsible for appearance of these DCs in the steady-state.

Immediately after subcutaneous injection of soluble protein (FITC-OVA), immature DCs were much more efficiently labeled with FITC (maximum on 2 hours after injection) than semi-mature ones, and antigen presentation became detectable 4 hours after injection. These results indicate that immature DCs are resident in the LNs and work for uptake and presentation of lymph-derived soluble antigens in the inflammatory condition. In contrast, semi-mature DCs are considered to transfer self-antigens from the periphery and present them in the steady state.

After painting hapten (FITC or TRITC) dissolved in irritant aceton/dibutylphtalate, only fully-mature DCs were strongly labeled with hapten (maximum on 48 hours after paint) in the draining LNs. Immediately after paint, immature DCs were more efficiently but weakly labeled with hapten only when quite high dose of hapten was applied. In a dose which is usually used for induction of contact hypersensitibity (5mg/ml), hapten-labeled fully-mature DCs appeared only since 24 hours after paint, indicating that these DCs are newly-migrated ones transferring antigen from the skin. These hapten-labeled fully-mature DCs were distinguished from unlabeled semi-mature ones by higher expression levels of MHC class II and B7-2 but lower expression of CCR7. The same results were observed when the DCs obtained by organ culture of split ears were ex vivo stimulated with anti-CD40 antibody.

Thus, we can distinguish immature resident, semi-mature migratory and fully-mature newly-migrated DCs in the skin-draining LNs.

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HHV8 Infection of Umbilical Cord-Blood-Derived CD34+ Stem Cells Enhances the Immunostimulatory Function of their Dendritic Cell Progeny

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Kaposi's sarcoma-associated herpesvirus (KSHV) also called human herpesvirus 8 (HHV8) is a gamma-2 herpesvirus and was first described in Kaposi's sarcoma (KS) of AIDS-patients. HHV8 is detected regularly in biopsies of all clinical forms of KS, classic, AIDS-related, endemic, and post-transplantation, in spindle cells in the dermis, but also in the lungs, liver, and intestine. Spindle cells are thought to originate from circulating peripheral blood haemopoietic precursor cells. CD34+ stem cells carrying HHV8 have been described in the peripheral blood of AIDS patients suffering from Kaposi's sarcoma.

In this study we investigated the influence of HHV8 on the differentiation of CD34+ stem cells. Native CD34+ cells derived from umbilical cord blood could be infected by a laboratory strain of HHV8, as shown by immunofluorescence staining, PCR and transmission electron microscopy, but no significant initial maturation/differentiation effects were observed. In immunofluorescence experiments these infected cells were shown to differentiate into immature and mature dendritic cells (DC) following cytokine induction with rhGM-CSF, rhTNF-alpha and rhSCF which was demonstrated by upregulation of CD83 and CD86 molecules. Subsequently the immunostimulating capacity of DC populations was tested in a mixed lymphocyte reaction using allogenic T cells. The HHV8-infected CD34+ progenitor cell-derived mature DC population showed a significantly enhanced antigen presenting capacity as compared to not-infected DC, which was not observed with the immature DC. This suggests stimulation of DC function by HHV8 infection. Since there is only a small percentage of HHV8 positive DC in the preparations and it is not clear whether infection is abortive or productive to some extent, this seems to be most likely due to an indirect viral effect.

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Molecular Cytogenetic Analysis of Chromosomal Breakpoints in the *IGH, MYC, BCL6* and *MALT1* Gene Loci In Primary Cutaneous B-cell Lymphomas

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Chromosomal translocations affecting the *IGH* locus and various oncogene loci are recurrent in many types of systemic B-cell lymphomas. However, hardly any data exist on such translocations in primary cutaneous B-cell lymphomas. Here, a series of 29 primary cutaneous B-cell lymphomas was investigated by interphase fluorescence *in situ* hybridization with probes for the *IGH, MYC, BCL6 and MALT1* loci. None of 6 follicle center cell lymphomas and 9 marginal zone lymphomas showed evidence for any translocation affecting these loci. In contrast, 11 of 14 large B-cell lymphomas of the leg harbored breakpoints in at least one of the loci. Translocations involving the *MYC* locus were detected in 6 cases, 5 of them derived from a *MYC/IGH* juxtaposition and 1 from a translocation involving a non-*IG* gene partner. Rearrangements of the *BCL6* locus were detected in 5 B-cell lymphomas of the leg, and involved *IGH* (2 cases), *IGL* (1 case) and non-*IG* genes (2 cases). The present study shows that large B-cell lymphomas of the leg display a pattern of chromosomal translocations similar to their systemic counterparts whereas primary cutaneous follicle center cell lymphomas and marginal zone lymphomas lack these typical chromosomal translocations.

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Tolerance to Intravenous Heparin in Patients with Delayed-type Hypersensitivity to Heparins: a Prospective Study

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In the course of subcutaneous heparin injections eczema-like plaques due to delayed-type hypersensitivity may occur. Is intravenous administration of heparin contraindicated or a safe alternative in these patients? 38 consecutive patients who were referred to our hospital for suspected hypersensitivity to subcutaneous heparins between 1st of January 1998 to 31st of December 2003 were subjected to a three-step diagnostic procedure including skin tests (intradermal and patch test), subcutaneous and intravenous challenges. 27 patients with delayed-type hypersensitivity accomplished all three diagnostic steps. Out of these 19 were skin test-positive, whereas in 8 skin test-negative patients delayed-type hypersensitivity to heparins was proven by subcutaneous challenge. Most important, the intravenous challenge test with heparin was tolerated uneventfully by all 27 patients. Therefore, in case of therapeutic necessity the simple shift from subcutaneous to intravenous administration of heparin is justified.

257 [Oral 060]

Timing of Exposure to Staphylococcal Enterotoxin B (SEB) is Critical to IgE Antibody Production in an Animal Model of Atopic Dermatitis

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Staphylococcal enterotoxin B (SEB) has been shown to cause exacerbation of atopic dermatitis. Bacterial products have also been shown to induce Th1-deviated response that could prevent Th2-mediated responses. An animal model of atopic dermatitis was used to study the effect of SEB on allergen-specific and total IgE. (1) Ovalbumin (OVA) was used to induce the model. BALB/c mice were epicutaneously sensitized for three one-week-long periods, separated by two-week intervals of no treatment, totalling 7 weeks. When treated topically with SEB during three one-week-long sensitization-periods of the model, both OVA-specific and total IgE were significantly and dose-dependently upregulated. (2) Animal model was induced with OVA without SEB application during first 7 weeks, and continued for an additional 6 weeks with two more one-week-long sensitization periods with OVA and with combination of OVA and SEB. After these treatments SEB significantly down-regulated both OVA-specific and total IgE levels. Bacterial superantigens may exacerbate IgE production during the development of atopic dermatitis and downregulate serum IgE-levels in already established atopic dermatitis.

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Chemokine Responses Distinguish Chemical-Induced Allergic From Irritant Skin Inflammation

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Chemical-induced adverse effects such as contact allergy as well as irritancy are of major importance in clinical dermatology and during the development of new pharmaceuticals or industrial chemicals. Since clinical and histological features of allergic and irritant contact dermatitis are similar, the differentiation between both types of dermatitis in the preclinical and clinical evaluation of chemicals remains difficult. However, the underlying immunological mechanisms are thought to be fundamentally different. Here we systematically studied the involvement of chemokine superfamily members ($n = 45$) in the pathogenesis of both chemical-induced allergic and irritant contact dermatitis in mice and humans. Chemokines are small cytokine-like molecules binding G-protein coupled receptors and have been shown to critically regulate leukocyte trafficking. Comprehensive analyses of the chemokine and chemokine receptor expression during chemical-induced allergic and irritant skin responses indicated that hapten-induced murine contact hypersensitivity represents a valid model for human allergic contact dermatitis at the molecular level when compared with nickel sulfate patch test or genuine allergic contact dermatitis lesions. Notably, the expression of CXCR3 ligands such as CXCL9 *(Mig)*, CXCL10 *(IP-10)* and CXCL11 *(ITAC)* distinguished between chemical-induced allergic and irritant skin responses in both murine and human models of contact dermatitis. Furthermore, we could show that these inflammatory chemokines synergize with the homeostatic chemokine CXCL12 *(SDF-1)* in recruiting skin homing CLA+ memory T cells. Taken together, findings of the presents study provide important insights into the molecular basis of chemical-induced allergic and irritant contact dermatitis, identify novel markers for their differentiation and demonstrate the cooperation of inflammatory and homeostatic chemokines in the recruitment of pathogenic leukocyte subsets.

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Imaging of Angiogenesis in Delayed Type Hypersensitivity Reactions by Positron Emission Tomography and [18F]Galacto-RGD

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Angiogenesis plays a major role in organ-specific autoimmune diseases caused by delayed type hypersensitivity reactions. Angiogenesis leads to an increase in the cellular traffic from the blood stream to inflammatory tissue sites and is required for chronicity. Vascular cell integrin $\alpha_v\beta_3$ mediates cellular adhesion to extacellular matrix proteins and is selectively induced during angiogenesis. To better understand mechanisms of chronic inflammation, we investigated the role of $\alpha_v\beta_3$-integrin in recurrent episodes of cutaneous DTHR using RGD-peptide that selectively binds $\alpha_v\beta_3$-integrin. Mice were sensitized and challenged with trinitrochlorobenzene (TNCB) to induce and elicit contact hypersensitivity reactions (CHSR). To investigate T cell independent cutaneous inflammation mice were challenged with phorbol myristate acetate (PMA). 12h after TNCB or PMA challenge animals were injected with [^{18}F]Galacto-RGD or [^{125}I]Gluco-RGD peptide and scanned *in vivo* with the small animal positron emission tomograph MADPET or uptake was determined by autoradiography. *In vivo* MADPET images confirmed intense RGD peptide uptake after the second TNCB-challenge but not after a single TNCB-challenge. No uptake was determined in mice pretreated with unlabeld RGD peptide. The uptake ratio right ear (treated) versus left ear (untreated) was 2.9 after the 8th TNCB-challenge, no increase was detected after a single TNCB-challenge. Poor RGD peptide uptake was observed after the 8th PMA challenge compared to TNCB-challenged littermates. In contrast to PMA, TNCB-specific CHSR showed a decreased RGD-peptide binding after the 8th TNCB-challenge. H&E stained sections confirmed an enhanced angiogenesis in chronic CHSR. Thus, angiogenesis and $\alpha_v\beta_3$-integrin play an essential role in chronic CHSR. Most important chronic hapten-specific CHSR is more efficient in inducing $\alpha_v\beta_3$-integrin expression compared to antigen independent chronic inflammation. Our data represent a new model to investigate angiogenesis in chronic antigen specific T cell mediated immune responses such as atopic dermatitis or psoriasis.

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Sweat Induces IgE-mediated Allergic Reaction in Patients with Atopic Dermatitis

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Sweating has been recognized as one of the aggravating factors in atopic dermatitis (AD), but the mechanism of such aggravation is unclear. We have recently demonstrated that 84% of patients with AD showed positive skin reactions to autologous sweat samples, whereas only 11% of healthy subjects did so, suggesting that constituent(s) of sweat can provoke allergic reactions selectively in patients with AD. In this study, we investigated the specificity of sweat-induced reaction in *in vitro* basophil-histamine release tests and demonstrated crucial involvement of IgE in this reaction. Sweat samples were collected from healthy volunteers, pooled and purified by combination of chromatographies. The partially purified sweat preparation induced histamine release from basophils of 50 of 66 (74.6%) patients with AD, three of 28 (10.7%) healthy controls and none of 13 patients with psoriasis vulgaris showed positive reaction. To know the mechanisms of sweat-induced histamine release from basophils of the patients with AD, we purified IgE from the serum of a patient with AD and examined its capability of mediating sweat-induced reaction in human mast cell line (LAD2), rat basophil leukemia cell line transfected with human α-subunit of the high affinity IgE receptor (RBL48), and human basophils of healthy volunteers. These cells showed degranulation upon stimulation with the semi-purified sweat preparation when sensitized with the patient-derived IgE, but not when sensitized with myeloma IgE, thus indicating the presence of specific IgE against sweat antigen(s) in sera of patients with AD. These results demonstrate that sweat may induce allergic reactions in patients with AD, resulting in the aggravation of dermatitis. Sweat allergy should be a useful marker for the diagnosis of AD, and a target for new therapeutics.

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A Diminished Th2 type but Enhanced Th1 type Response in the Atopic Dermatitis Mouse Model with CCR5−/− Mice

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Clinical features of atopic dermatitis (AD) involve elevated total and allergen-specific IgE levels, increased eosinophilia and Th2 type cell infiltration in the lesional areas of the skin. As the disease becomes chronic, Th1-cells start to dominate in eczematous skin lesions. The CC chemokine receptor 5 (CCR5) is expressed preferentially on Th1 cells. We used CCR5 deficient mice (CCR5−/−) to assess the role of CCR5 in a murine model of atopic dermatitis induced by repeated epicutaneous sensitization with ovalbumin (OVA), and characterized by eosinophil skin infiltration, local expression of Th2 cytokines and elevation of IgE antibodies. Infiltration in CCR5 mice by mononuclear cells and eosinophils in OVA-sensitized skin was comparable to that in wild-type (WT) controls. However, sensitized skin of CCR5−/− mice had decreased expression of Th2-type cytokines (IL-4, IL-5 and IL-13) but increased expression of Th1 cytokines (IFN-gamma and IL-18) compared to WT controls. Moreover, expression of CCL27 and CCL20 was elevated in sensitized skin sites of CCR5−/− mice compared to WT. Finally, allergen sensitized CCR5−/− mice produced degreased levels OVA-specific IgE compared to WT. These results suggest that CCR5 plays significant role in the regulation Th1/Th2 balance in the skin and circulating allergen-specific IgE levels in an animal model of atopic dermatitis.

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Stress Induces Plasticity of the Peripheral Peptidergic Innervation and Exacerbates Neurogenic Inflammation: Pathomechanisms in Atopic Dermatitis?

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Stress is said to induce itchiness of the skin and exacerbate inflammatory skin diseases such as atopic dermatitis. In this context, stress mediators such as the neuropeptide substance P (SP) play a role as immunmodulators and in a wider sense growth factors. E.g. we were recently able to show, that stress or treatment of mice with SP is associated with mast cell degranulation, increased cutaneous inflammation and increased apoptosis in the hair follicle. However, local interactions between the nervous and immune systems, especially under perceived stress, have rarely been reported. Here we show, that sonic stress exposure increases the number of SP-immunoreactive nerve fibres in the back skin of C57BL/6 mice with all there hair follicles in the resting phase of the hair cycle (telogen – low numbers of cutaneous nerve fibers). Such SP immunoreactive nerve fibres contacted mast cells more frequently. At the same time the percentage of degranulated mast cells increased and apoptotic cell number in the skin was upregulated. By semiquantitative rtPCR C57BL/6 mice with experimentally induced allergic dermatitis showed increased cutaneous IL-4 and to a lesser degree IFN-γ production in the skin 48hrs after stress exposure and induction of allergic dermatitis. Stress only mildly increased IL-4 but increased dermal infiltration e.g. by eosinophils in allergic dermatitis mice over mice that were either stressed or had allergic dermatitis as well as over untreated controls. Increased infiltration was associated with epidermal thickening, and more VCAM-immunoreactive blood vessels. At the same time the percentage of degranulated mast cells increased significantly and the number of SP-immunoreactive peptidergic sensory nerve fibers decreased in the acute allergic dermatitis lesions. Ultrastructural investigation showed unmyelinated peptidergic nerve fibres in a state of deterioration close to degranulating mast cells and eosinophils in the skin of stressed mice with allergic dermatitis, suggesting a decreased number of SP-immunoreactive nerve fibres due to active release of SP in the course of neurogenic inflammation. This may lead to an upregulation of endothelial adhesion molecules and increased infiltration by immunocytes to the skin. These data provide first evidence for stress-induced exacerbation of cutaneous allergic diseases such as atopic dermatitis by local interaction of the peripheral nervous system with the immune system.

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A Hypoallergenic Recombinant Allergen-Derivative of the Major Timothy Grass Pollen Allergen, Phl p 2 Obtained by a Mosaic Strategy

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Summary. Approximately 200 million allergic patients are sensitized to group 2 grass pollen allergens. Here we report the development of a recombinant Phl p 2 allergen derivative with reduced allergenic activity for immunotherapy of grass pollen allergy using a novel mosaic approach.
Methods & Results. First we have disrupted the three-dimensional β-sheet structure of the 10 kDa Phl p 2 molecule by synthesizing three peptides comprising the first, second and third part of Phl p 2. Testing with IgE from Phl p 2 allergic patients and basophil histamine release experiments showed that the three peptides had lost their IgE reactivity and allergenic activity. Next we built an artificial Phl p 2 mosaic molecule by PCR-based gene soeing of cDNAs coding for the three peptides in altered order. Circular dichroism experiments showed that the recombinant Phl p 2 mosaic molecule had an altered three-dimensional structure compared to Phl p 2 wildtype and showed a more than 100-fold reduced allergenic activity. Immunization of rabbits with the rPhl p 2 mosaic induced IgG antibodies which inhibited the binding of allergic patients IgE antibodies to the Phl p 2 wildtype allergen.
Conclusion. The Phl p 2 mosaic molecule is a candidate molecule for immunotherapy of grass pollen allergy and the mosaic technology a general strategy for the production of allergy vaccines.

Supported by grants F01801, F01805, F01809, Y078GEN of the Austrian Science Fund, by the CeMM project of the Austrian Academy of Sciences and by a research grant from BIOMAY, Vienna, Austria.

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Long-lived Th2 Immunological Memory in B cell Deficient Mice

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Elucidating the mechanisms underlying the maintenance of CD4 T cell memory is important for developing improved immunomodulation therapies aimed at enhancing memory responses to pathogens and inhibiting memory responses to allergens, self, and transplanted tissue. Although the factors involved in maintaining CD4 T memory cell survival are unknown, there is evidence that B cells may be necessary. We chose to test the role of B cells on Th2 memory using a mouse model of allergic asthma in which mice develop acute onset disease upon exposure to soluble followed by nebulized antigen. These mice recover within 30 days of acute disease but maintain chronic lung infiltrates consisting of CD4 T cells, B cells, and macrophages for over 800 days after acute disease. Existence of memory CD4 T cells in recovered mice is demonstrated by lifelong immune responses to aerosolized antigen leading to lung eosinophilia, mucus hypersecretion, and elevated antigen-specific IgE. Lung CD4 T cells from recovered mice respond to antigen *in vitro* and upon adoptive transfer can induce allergic asthma in naïve mice following aerosol antigen. To test whether B cells play a role in the maintenance of long-lived memory Th2 cells, we induced acute allergic asthma in B cell deficient mice and over 1 year later, evaluated recall responses to aerosol antigen. We observed that mice lacking B cells had acute disease similar to wild type mice, excluding the presence of B cells in lung infiltrates and immunoglobulin. The B cell deficient mice maintained lung infiltrates containing memory Th2 memory cells, 1 year after recuperating from acute disease. Furthermore, recovered B cell deficient mice developed disease relapse characterized by allergic eosinophilic lung inflammation and mucus hypersecretion upon aerosol antigen challenge. These data demonstrate that B cells are not necessary for the maintenance of long-lived Th2 memory.

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The Dendritic Cell Repertoire of Atopic Dermatitis: Evidence for Two Non-Indigenous Cell Populations

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Because of their unique ability to initiate primary immune responses in T- lymphocytes, dendritic cells are thought to play a key role in the pathogenesis of atopic dermatitis. To learn more about the process initiating and propagating the cutaneous allergic reaction, we phenotypically analyzed various dendritic cells subsets in both skin biopsies (n = 6; n = 5) and peripheral blood (n = 10; n = 8) of atopic dermatitis patients and healthy controls. Our findings confirm the presence of resident types of dendritic cells in normal human skin, namely epidermal CD1a$^+$/Langerin$^+$ Langerhans cells and CD1c$^+$ dermal dendritic cells. In atopic dermatitis lesions two non-indigenous dendritic cell subsets occurred besides increased numbers of Langerhans cells and dermal dendritic cells: (i) CD1a$^+$/FcεRI$^+$/CD1c$^+$ dendritic cells in the epidermis (inflammatory dendritic epidermal cells) and dermis and (ii) CD123$^+$/BDCA-2$^+$/CD45RA$^+$/CD68$^+$ cells in the dermis. These latter cells exhibit the phenotypic features of plasmacytoid dendritic cells and, interestingly enough, display FcεRI on their surface. A detailed characterization of dendritic cell subsets in the skin and in the peripheral blood including their activation status and their ability to bind IgE should form the basis for the investigation of dendritic cell-mediated events operative in the pathogenesis of the eczematous response.

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***N-acetyltransferase 2* Acetylation Genotypes and Serum IgE Levels**

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IgE mediated allergic reactions play a crucial role especially in industrialized societies. A role of the *N-acetyltransferase 2* (*NAT2*) phenotype and genotype has been investigated previously in allergic diseases such as food allergies, allergic rhinitis, and atopic asthma. We studied the *NAT2* genotypes in persons with 'high' IgE levels. In 218 cases with high total serum IgE-levels (>200 IU) and 192 controls the genotypes coding for slow and fast acetylator *NAT2* phenotypes were identified by real-time polymerase with hybridization probes. Mutations at positions 341 (C -> T), 481 (C -> T), 590 (G -> A) and 857 (G -> A) of the *NAT2* gene were examined. The acetylation phenotype based on genotyping was defined as the occurrence of at least one wild type allele. Allele frequencies were calculated according to the Hardy-Weinberg equilibrium. Statistical analysis showed that 39.9% of the cases and 47.4% of the controls were fast acetylators based on genotyping, while 60.1% of the cases and 52.6% of the controls were classified as slow acetylators. Five cases (2.3%) and 16 controls (8.3%) were classified as wildtypes, whereas 59 cases (27.1%) and only 31 controls (16.1%) had a homozygous mutation at nucleotide position 481 (C -> T). Allele frequencies for cases were: *NAT2*4* (wild type): 17.2%, **5A/B*: 48.9%, **5C*: 5.7%, **6A/B*: 25.9% and **7A/B*: 2.3%. Allele frequencies for controls were *NAT2*4*: 25.6%, **5A/B*: 42.4%, **5C*: 4.7%, **6A/B*: 24.7% and **7A/B*: 2.6% compared favourably to published data. In contrast, we found in the group with high total serum IgE levels a higher frequency of homozygous mutations at position 481 (C -> T) compared to controls. These results suggest that certain mutations in the *NAT2* gene may be associated with high total IgE levels.

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Anti-Histamines: Implication as Immune Modulators

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To date, it has been convinced that only immuno-pharmacological mechanism of anti-histamines is its competitive inhibition to the type 1 histamine receptor (H1R). However, their continuous use often results in prolonged remission of allergic skin disorders, such as atopic dermatitis. Th1 type-immunity is thought to constitute pathology of acute deterioration phase, while Th2-type immune response plays a causal role in its chronic stage. Interleukin (IL)-4 and IL-12 is well known to initiate Th2 and Th1-type immune response, respectively. Hence, this attempts us to examine modulatory impacts of anti-histamines on cellular events induced by these cytokines. To address this issue, human peripheral blood-derived T cells were isolated via density gradient centrifugation followed by nylon wool column-purification, leading to recovery of 95% pure T cells. T cells were then pretreated for 15 min with various concentrations of a different kind of anti-histamines (cetirizine, fexofenadine, loratadine and olopatadine) and stimultated with 1–10 ng/ml recombinant human interleukin 4 (rhIL-4) or 1 ng/ml rhIL-12. Cells were harvested 3 hours poststimulation, and RNA was isolated, reverse-transcribed and semi-quantitative RT-PCR performed using primers specific for IL-5 or interferon (IFN)-γ. While fexofenadine and loratidine downregulated IL-12-induced expression of IFN-γ mRNA, cetirizine and olopatadine failed to do so. By contrast, all anti-histamines examined suppressed IL-5 mRNA expression upregulated by IL-4. To evaluate the effects at a protein level, cultured supernatants were collected 24 hours after stimulation, and ELISA assays were performed, demonstrating that cetirizine or fexofenadine, but not loratadine or olopatadine suppressed IL-5 protein expression induced by IL-4. By contrast, all anti-histamines except for cetirizine significantly suppressed IL-12-enhanced release of IFN-γ. Together, our present study demonstrates that in addition to conventional view, anti-histamines may differently modulate Th1 or Th2 type immune response. Furthermore, this might suggest that disease states should be taken into account in order to more efficiently treat allergic skin disorders by anti-histamines.

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Levocetirizine Decreases VCAM-1 Expression Induced by TNF-alpha in Human Dermal Endothelial Cells Through Down-Regulation of NF-kappaB Pathway

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The expression of adhesion molecules by endothelial cells is necessary for the recruitment of eosinophils from the bloodstream to the skin and its pharmacological alteration might down-regulate local tissue eosinophilia and persistence of allergic inflammation. In the present work, we investigated the modulatory effect of levocetirizine on the regulation of the expression of vascular cell adhesion molecule-1 (VCAM-1) in human dermal endothelial cells (HDMEC) in response to cytokines *in vitro*. Since the regulation of adhesion molecules expression involves the nuclear factor kappa B (NF-kB) pathway, we evaluated the possible effect of levocetirizine on the NF-kB activation *in vitro*. HDMEC, obtained from normal adult breast skin, and a SV40-transformed cell line (HMEC-1), were treated with levocetirizine (10^{-7} to 10^{-4} M, maximal 24h) before they were stimulated with 10–100 U/ml TNF-α for 4 to 24 h. NF-kB activity was measured with the electrophoretic mobility shift assay (EMSA) and by reporter gene analysis after specific transfection with NF-κB–luciferase plasmid reporters. The cellular localization of NF-κB proteins was analysed by immunocytochemistry and confocal microscopy. To further analyze the mechanisms of levocetirizine effect on NF-κB pathway, the expression of cytoplasmic NF-κB inhibitors (IκB) was investigated by immunoblotting. The synthesis and expression of VCAM-1 was studied by semi-quantitative RT-PCR, immunoblot, immunocytochemistry and flow cytometry. Our results clearly showed that the potent nuclear translocation of NF-κB induced by TNF-α in HDMEC was inhibited by levocetirizine in a dose-dependent fashion, as shown by EMSA and cytochemistry. There was no basal level of NF-κB in untreated HDMEC or in cells exposed to levocetirizine only. Levocetirizine decreased VCAM-1 mRNA levels and VCAM-1 expression at the surface of HDMEC, in a dose-dependent fashion from 10^{-4} to 10^{-7} M. This range of concentrations may be found *in vivo*. This inhibitory effect may not be mediated through H1-receptors since TNF-α alone was used as the stimulant for VCAM-1 up-regulation. In conclusion, these data support previous work on anti-inflammatory effects of levocetirizine, which appear independent of H1-receptor blockade, and suggest a possible mechanism whereby this new anti-H1 may inhibit eosinophil accumulation *in vivo*.

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Anaphylaxis to Magnetic Resonance Contrast Media due to Type-I-Allergy to Meglumine

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Acute reactions to iodinated contrast media are common and considered pseudoallergic due to non-specific complement activation, release of histamine and other mediators. In contrast, reactions to Magnetic resonance (MR) contrast media (gadolinium chelates) are very rare, and little is known about underlying mechanisms.

Case report. a 23-year old female patient underwent MRI because of being suspected of osteoid osteoma. Within minutes after application of gadoterate meglumine (DOTAREM®), she developed an anaphylactic reaction grade III according to Muller, with generalized urticaria, bronchospasm and drop of blood pressure. She fully recovered after antiallergic treatment at the intensive care unit of the hospital. The patient had tolerated iopromide (ULTRAVIST®) earlier, but reported pruritus after gadopentetate dimeglumine (MAGNEVIST®) several years before.

Test results. Skin prick-test (SPT) showed highly positive reactions to Dotarem® and other contrast media and chemically different drugs (Micardis® and Novonorm®) containing meglumine. Contrast media not containing meglumine were negative. *In vitro* evidence of an IgE-mediated activation of basophiles by CD203c activation test is in preparation.

Discussion. Meglumine (also methylglucamine; CA Index Name D-Glucitol, 1-deoxy-1-(methylamino)-(9Cl)) is used for stabilisation and reduction of toxicity of the gadolinium compound in MR contrast media. It is also found as adjuvant in other pharmaceutical products and meglumine antimonate is used in the treatment of leishmaniosis. Anaphylactic reactions due to meglumine have not been described yet. The severe clinical picture and the positive skin prick tests suggest an IgE-mediated mechanism.

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Double-Blind Randomised Trial Evaluation of a New Product Containing Mmp and Pkc Inhibitors in Cutaneous Contact Hypersensitivity

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Matrix metalloproteases (MMPs) and PKC are implicated in several skin pathologies, especially inflammatory cutaneous disorders such as psoriasis and contact hypersensitivity. More over, several molecules regulate the migration of skin dendritic cells, which are potent antigen presenting cells implicated in the primary T-cell-mediated immune response of the skin, among which MMPs and PKC. We have previously shown that MMP and/or PKC inhibitors are able to modulate the migration of skin dendritic cells both *in vitro* and *in vivo*. Thus the aim of this study was to evaluate the clinical efficiency of an innovative topical product containing both MMPs and PKC inhibitors (verum) on cutaneous contact hypersensitivity (CHS).

A double-blind randomised trial was conducted by 10 dermatologists on 36 selected volunteers (mean age 36 years) having repeated nickel allergy due to buckles. The panellists have applied products on both ears (18 : placebo or 18 : verum) twice a day during 7 days, and then buckles until CHS appears (at least for 14 days, 8 to 12h per day). An individual board has to be filled by the panellist in order to assess the course and development of CHS. At day 14, a final examination was performed by the dermatologist.

The first results showed that the preventive application of the verum allows to double the time of buckles wearing compare to the placebo (6 days/13 days) and also to decrease more significantly (vs placebo) the clinical signs of CHS.

In this trial, the verum has been proven to be more efficient than the placebo in preventing and/or delaying CHS as well as in reducing its the clinical signs. This study underline for the first time the effectiveness of MMP and PKC inhibitors in the management of allergic skin.

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Detection of Serum IgE Auto-Antibodies Against Epidermal Proteins in Severe Forms of Atopic Dermatitis

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Many symptoms of atopic diseases result from events initiated by cross-linking of cell-bound IgE specific for exogenous allergens. Previous studies have shown that sera of patients with severe atopic dermatitis (AD) may also contain IgE specific for self-proteins, supporting the hypothesis of autoreactivity as pathogenic factor. In these studies IgE-autoreactivity was detected against largely undefined proteins of an epithelial cell-line, cultured fibroblasts, endothelial cells and peripheral blood-derived cells. Thus it remains unknown if sensitization to skin-derived self-proteins may occur in atopic dermatitis. Addressing this question we analyzed the sera of 202 atopic dermatitis patients, 36 patients with psoriasis and 26 healthy donors for specific IgE antibodies against protein-extracts of normal epidermis, dermis and the epithelial cell-line A431. Whereas only serum of one psoriatic patient and no sera from healthy persons showed specific reactivity against A431-derived proteins, 21% of the atopic dermatitis patients exhibited IgE-reactivity, thus confirming previous findings. Importantly, 23% of the AD patients revealed serum IgE with specificities against epidermal-extracts. These proteins differed in their molecular weights from those identified in A431-extracts indicating distinct IgE specificities. In contrast to the epidermis no IgE reactivity was detectable against dermal protein-extracts in all sera tested.

These data demonstrate the presence of auto-reactive IgE antibodies against epidermal self-proteins in a subgroup of patients with severe atopic dermatitis, and suggest that IgE autoreactivity may function to maintain/augment the cutaneous allergic reaction.

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Expression of the Non-Receptor Associated Tyrosine Kinase p72syk Influences the Metastatic Behaviour of Melanoma Cells

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Once human melanoma has spread to distant sites and has set metastases, every treatment so far is primarily palliative. Therefore insight into the metastatic process in melanoma is very important. p72syk (spleen tyrosine kinase), a member of the group of non-receptor associated tyrosine kinases is well described as a signal transducing protein downstream of immunoreceptors and integrins. In mammary malignancies, the absence of syk was shown to be associated with an increased metastatic potential of the tumor cells. Based on this data we investigated the expression of syk in melanoytes and melanoma cells and assessed the impact of syk-expression on the metastatic behaviour of melanoma cells. Syk was expressed in different batches of normal human adult epidermal melanocytes but was not expressed in any of the melanoma cell lines tested. Plasmid driven expression of syk in melanoma cells (Plasmids courtesy of Dr. S Mueller, Washington, DC) did not have any significant impact on the growth speed of melanoma cells in a normal culture dish. In contrast, migration through an artificial basement membrane layer (Matrigel-Assay, BD), was highly reduced in melanoma cells expressing syk after transfection, as compared to wild type or control vector cells. Injection of syk transfected melanoma cells in SCID mice led to a significant delay in tumor take after subcutaneous injection and to a highly decreased number of metastatic tumot lesions after injection into the tail vein. This data clearly positions syk as an important factor influencing the metastatic behaviour of human melanoma cells.

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Tetraspanins are Implicated in Melanocyte Adhesion and Motility

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Tetraspanins have been implicated in regulation of cell development, proliferation, activation and motility and have been shown to couple to signal transduction pathways. CD9 may regulate melanoma invasion. We have explored the role of tetraspanins CD9, CD81 and CD151 in adhesion and migration of normal human melanocytes.

We performed adhesion experiments to collagen and fibronectin to study the influence of tetraspanins in melanocyte adhesion. We found a stimulation of adhesion to collagen using TS2/16 (anti-CD29 that activates β1 integrins) and inhibition using VJ1/14 (anti-CD29 that inhibits β1 integrins). When we incubated melanocytes with anti-tetraspanin monoclonal antibodies, we found an inhibition of adhesion in collagen at 20 min with CD9, CD81 and CD151. When we incubate melanocytes with the monoclonal antibody for 1 hour, we found an enhance of melanocyte adhesion with the anti-tetraspanin monoclonal antibodies,

On the other hand, we did not found any effect of anti-tetraspanin monoclonal antibodies in adhesion to fibronectin, although statistical differences were found between TS2/16 and VJ1/14.

We used the trasnwell migration assay to evaluate the influence of monoclonal antibodies in melanocyte motility. We found that TS2/16 did not modify the motility of melanocytes while VJ1/14 stimulates migration of melanocytes. Monoclonal antibodies against tetraspanin molecules showed different behaviors. CD9 and CD151 enhanced melanocyte motility, while CD81 did not modify the migration of melanocytes.

Published results suggest that tetraspanin are not involved in cellular adhesion in most cells. We have found that tetraspanins CD9, CD81 and CD151 inhibited adhesion to collagen at 20 minutes and enhanced it at 1 hour. This behavior has not been previously described for tetraspanins in other cells.

We have found that anti-CD9 enhance the motility of melanocytes in the transwell migration assay. In human melanoma cells expressing high levels of CD9, anti-CD9 slightly inhibit transwell filter migration although anti-CD9 presented a strong inhibitory effect of transendothelial migration. In human and mouse melanoma cells, low CD9 expression has been related to enhanced motility, and transfection of mouse melanoma cells with CD9 lowered their metastatic potential and mobility. These results imply CD9 in the migration of melanocytes and melanoma cells but its function is not clear. Anti-CD151 enhances the motility of melanocytes in the transwell migration assay. In contrast, anti-CD151 inhibited keratinocyte and endothelial migration in the wound-healing assay. Although anti-CD81 inhibited keratinocyte motility, no effect was found on melanocytes or endothelial cells.

It seems clear that each tetraspanin has different role in each tissue, and its functions are specifically modulated.

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Leukocyte Adhesion to Activated Endothelial Cells Mediated by the Interaction Between Thy-1 (CD90) and Mac-1 (CD11b/CD18) – a Competition for ICAM-1?

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Human Thy-1 was shown to be a cell adhesion molecule expressed on the cell surface of activated endothelial cells. In previous studies we could demonstrate, that human Thy-1 interacts with a corresponding ligand on the cell surface of polymorphonuclear cells (PMNC) and monocytes, and of melanoma cells as well, mediating the adhesion of both leukocytes and melanoma cells to activated human dermal microvascular endothelial cells (HDMEC) expressing Thy-1. While the leukocyte integrin Mac-1 ($\alpha_M\beta_2$, CD11b/CD18) was determined as the counterreceptor of Thy-1 on human leukocytes, the corresponding ligand of Thy-1 expressed on melanoma cells was still unknown.

In this study we could demonstrate an interaction between human Thy-1 and the integrin $\alpha_V\beta_3$ (CD51/CD61) by the binding of purified human Thy-1 to $\alpha_V\beta_3$-transfected cells as well as by the specific interaction between the purified proteins Thy-1 and $\alpha_V\beta_3$ in a cell-free system. Since the expression of $\alpha_V\beta_3$ is known to promote invasion and metastasis of melanoma cells, we studied the functional role of Thy-1 / $\alpha_V\beta_3$ interaction in cell adhesion of melanoma cells. Thus, we could observe an adhesion of the melanoma cell line JPC to Thy-1 transfected cells, that is inhibited by the previous binding of monoclonal antibodies against $\alpha_V\beta_3$ to the cell surface of JPC cells.

In conclusion these results indicate an interaction between human Thy-1 and the integrin $\alpha_V\beta_3$, mediating the adhesion of melanoma cells to Thy-1 transfectants and to activated endothelial cells expressing Thy-1. Thus, the cell adhesion molecule Thy-1 was demonstrated as a multi-ligand receptor interacting at least with two integrins – Mac-1 expressed on the cell surface of leukocytes and $\alpha_V\beta_3$ on melanoma cells as well. These various interactions suggest, that human Thy-1 possibly plays an important role not only in leukocyte extravasation into perivascular tissue, but also in melanoma cell invasion and metastasis.

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Narrow-Band UVB Stimulates Proliferation and Migration in Cultured Melanocytes

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Narrow-band UVB radiation is an effective treatment for vitiligo vulgaris. However, the mechanisms of narrow-band UVB in inducing repigmentation of vitiligo lesions are not thoroughly clarified. The purpose of our study was to investigate the effects of narrow-band UVB irradiation on melanocyte proliferation and migration *in vitro*. Our results showed that the cell counts as well as [^{3}H]thymidine uptake of melanocytes were significantly enhanced by narrow-band UVB irradiated keratinocyte supernatants. In these supernatants, a significant increase in basic fibroblast growth factor (bFGF) and endothelin-1 (ET-1) release was noticed. Basic fibroblast growth factor is a natural mitogen for melanocytes, whereas ET-1 can stimulate DNA synthesis in melanocytes. This stimulatory effect of melanocyte proliferation by supernatants derived from narrow-band UVB irradiated keratinocytes was significantly reduced by a selective endothelin B receptor antagonist (BQ788), suggesting an essential role of ET-1 on melanocyte proliferation. Our results of time-lapse microphotography revealed a stimulatory effect of narrow-band UVB irradiation on melanocyte migration. Focal adhesion kinase (FAK) plays a pivotal role in cell migration. Phosphorylated focal adhesion kinase ($p125^{FAK}$) expression on melanocyte was enhanced by narrow-band UVB irradiation. In this study, narrow-band UVB irradiation stimulated a significant increase in MMP-2 activity in melanocyte supernatant. Narrow-band UVB irradiation induced-migration of melanocytes was significantly annihilated by addition of $p125^{FAK}$ inhibitor (herbimycin A) or MMP-2 inhibitor (GM6001). These results suggest that $p125^{FAK}$ and MMP-2 activity play important roles in narrow-band UVB induced-migration of melanocytes. Our results provide a theoretical basis for the effectiveness of narrow-band UVB irradiation in treating vitiligo.

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Investigation of Proliferation and Pigmentation in Normal Human Adult Epidermal Melanocytes Cultured in Chemical-Free Medium

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Culturing of human epidermal melanocytes is a well-known method since 1982. The standard way of *in vitro* culturing for normal human melanocytes includes the use of chemical mitogens, such as the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) and the cyclic adenosine 3', 5' monophosphate (cAMP) enhancer cholera toxin (CT).

Previously we have established a new culture system that avoids chemical mitogens.

Using this more physiological *in vitro* environment, we aimed to investigate the effects of soluble factors (GM-CSF, IFN-γ, IL–1β, EGF and IL-10) and extracellular matrix proteins (fibronectin, collagen, laminin) on normal human adult melanocyte proliferation.

All the examined matrix proteins, and of the cytokines, GM-CSF, IL-1β and EGF enhanced melanocyte growth. We also found that autologous serum could replace the mitogenic effect of animal derived factors (FBS, BPE) and the chemical mitogen, TPA.

During *in vitro* culturing melanocytes loose their pigment production. With repeated UVB-irradiation (5.2 J/cm^2, four-times) or IFN-γ (1 ng/ml) addition cells regain pigmentation *in vitro*.

Because EGF exhibited a mitogenic effect on the melanocytes, we looked at the EGF receptor mRNA expression. Melanocytes expressed EGF receptor mRNA. EGF itself did not influence, but both repeated UVB-irradiation (5.2 J/cm^2)and IFN-γ (1 ng/ml) suppressed the expression of EGF receptor mRNA in cultured normal human adult epidermal melanocytes.

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Prevention of UV-Induced Immunosuppression by IL-12 is Dependent on DNA Repair

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Ultraviolet radiation (UV) suppresses the induction of contact hypersensitivity (CHS) and induces hapten-specific tolerance which is mediated via regulatory T cells (Tr). Interleukin (IL)-12 is known to prevent the suppression of CHS by UV and even to break UV-induced tolerance by yet unknown mechanisms. IL-12 recently was described to exhibit the capacity to reduce UV-induced DNA damage presumably via the induction of nucleotide excision repair (NER). UV-induced DNA damage is an essential molecular trigger for UV-mediated immunosuppression, thus we studied whether the restoring effect of IL-12 is linked to its capacity to reduce DNA damage. Injection of IL-12 into wild type mice (wt) which were sensitized through UV-exposed skin restored the CHS response completely. In contrast, *Xpa* knock out mice (*Xpa*−/−) which are deficient in NER were not able to mount an immune response after UV exposure despite administration of IL-12. This implies that the prevention of UV-induced suppression of CHS by IL-12 is dependent on properly functioning DNA repair. In contrast, IL-12 exhibited the capacity break already established UV-induced tolerance both in wt and *Xpa*−/−, indicating this effect to be independent of NER. Likewise, adoptive transfer of suppression via injection of Tr into naïve recipients was inhibited by IL-12 both in wt and *Xpa*−/−. Inhibition of sensitization by UV is due to the depletion of Langerhans cells (LC) which is triggered by UV-induced DNA damage. Accordingly, LC depletion by UV was prevented upon injection of IL-12 into wt but not in *Xpa*−/−. In addition, immunofluorescence staining revealed DNA damage carrying cells in the regional lymph nodes upon UV exposure. The number of these cells was remarkably reduced when UV-exposed mice had received IL-12. In turn, IL-12 did not reduce the number of DNA damage carrying cells in *Xpa*−/− mice. Taken together, these data indicate that the prevention of UV-induced inhibition of CHS by IL-12 is linked to its capacity to induce NER. In contrast, breaking of UV-induced tolerance and the activity of Tr by IL-12 is independent of NER and mediated via another yet to be determined mechanism. These data demonstrate for the first time a link between NER and the prevention of UV-induced immunosuppression by IL-12.

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Infusion of Apoptotic Cells by Photopheresis Induces Antigen-Specific Regulatory T Cells

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The basis of extracorporeal photopheresis (ECP) is the reinfusion of leukocytes, which have been exposed to 8-methoxypsoralen and UVA light. This treatment induces annexin V^+/$7AAD^-$ apoptosis of nearly all leukocytes. Photopheresis has shown evidence of benefit for the treatment of autoimmune diseases, solid organ transplant rejection and GvHD. The underlying immunological mechanism for its effect remains unresolved. Since UV radiation of the skin exhibits the capacity to induce tolerance via induction of antigen specific regulatory T cells, we studied whether ECP might induce a similar state of tolerance following extracorporeal treatment of leukocytes. For this purpose we utilized a murine model of contact hypersensitivity (CHS). Splenocytes and lymph node cells of mice, which were sensitized with dinitrofluorobenzene (DNFB), were exposed to experimental ECP *in vitro*. Experimental ECP-treated cells were injected intravenously into naive mice, which were subsequently skin sensitized with DNFB, and ear thickening measured following ear challenge. Animals, which had received experimental ECP-treated cells, were significantly suppressed in their CHS response. In contrast, mice which received cells which were untreated or exposed to UVA light or 8-MOP alone were not suppressed in their CHS response. Induction of suppression was lost when lymph node cells were depleted of $CD11c^+$ cells before experimental ECP treatment indicating that antigen presenting cells may be a primary target for photopheresis. This suppression was cell-mediated and antigen specific as demonstrated by the ability to transfer the tolerance to naive animals which could, however, properly respond to the unrelated hapten oxazalone. Transfer of tolerance was lost when cells were depleted of $CD4^+$ or $CD25^+$ subpopulations, indicating that experimental photopheresis induces regulatory cells, possibly of the CD4/CD25 lineage. As few as 100,000 unfractionated splenocytes could protect a naïve animal. The generation of increased number and activity of regulatory cells was evident when animals were boosted with antigen and this protection was dose dependent. Taken together, these data suggest that infusion of experimental ECP induced apoptotic cells produces highly active antigen specific regulatory cells. Further studies are underway to better understand the nature of these regulatory cells and their mechanism of induction.

279 [Oral 023]

Epidermal Turnover, an Alternative to UV-Induced Apoptosis in Removal of Arrested Cells

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The interplay between nucleotide excision repair (NER), apoptosis and cell cycle regulation in the UV-exposed epidermis is extremely important to avoid mutations and malignant transformation. In $XPC^{-/-}$ mice (deficient in NER in global genome) a cell cycle arrest of "near 4N" (sub-tetraploid) cells was observed peaking 72 hours after UV exposure (Van Oosten *et al*. PNAS, 2000). This arrest resolved without apoptosis (96–168 hrs). We surmised that these "near-4N"-arrested $XPC^{-/-}$ keratinocytes were removed from the epidermis by epidermal turnover.

A recently developed *in vivo* pulse-chase protocol, in which WT and $XPC^{-/-}$ mice subjected to 5 mg BrdU (i.p. T = 17) were sacrificed at various time points after UV exposure, was applied to determine the fate of BrdU-positive cells. Such a BrdU pulse, duration of 2 hours, enabled us to label 30% of the S/G_2-cells (DNA contents >2N) in $XPC^{-/-}$ 72 hours after UV exposure. From immunohistochemical analysis we concluded that epidermal turnover in WT and $XPC^{-/-}$ is completed within 7–10 days regardless of UV-exposure (no longer any suprabasal BrdU-positive or DNA-damaged cells present). Interestingly, a major increase in suprabasal cytokeratin 10-negative cells occurred only in UV-exposed $XPC^{-/-}$ mouse epidermis. In addition, we exclusively observed suprabasal cells positive for cytokeratin 5 (a basal cell marker) in UV-exposed $XPC^{-/-}$ epidermis. These observations clearly indicate an aberrant epidermal differentiation in XPC-deficient epidermis after single UV exposure. Flowcytometric analysis of single cell suspensions of UV-exposed $XPC^{-/-}$ epidermis further showed that the "near-4N" arrested cells retained cytokeratin 5. Combining these observations lead us to conclude that the arrested "near-4N" cells become detached from the basal layer without entering a proper differentiation programme, and are indeed subsequently lost through the epidermal turnover. This constitutes an alternative route, next to apoptosis, to eliminate DNA-damaged arrested cells from the epidermis.

280 [Oral 047]

Cockayne Syndrome A and B Proteins and Repair of Oxidative Damage to Mitochondrial DNA: Mechanism of Import and Functional Relevance *In Vivo*

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Cockayne Syndrome (CS), characterised by neurodegeneration, photosensitivity and premature aging, is caused by mutations in the CSA and CSB gene. We have previously shown that the CSA and CSB proteins are localised in the mitochondrion (mt) and involved in the repair of oxidatively induced mtDNA deletions. However, their mechanism of import into mitochondria, exact type of repaired damage and functional relevance *in vivo* have not been elucidated. Regarding localisation, both CSA and CSB miss a mitochondrial leader sequence but computer based k-NN sequence analysis predicted 52.2% and 30.4% mitochondrial localisation for CSA and CSB, respectively. Furthermore, co-immunoprecipitation showed requirement of CSA/CSB proteins in a heterodimeric state to allow mitochondrial import. Employing a reconstituted *in vitro* repair assay comprising fapy-glycosylase and endo III as reporter enzymes for oxidative damage in pyrimidines and purines, respectively, we could show that, after repetitive sublethal UVA irradiation, removal of thymine glycol from mtDNA was defective in fibroblasts from CS patients. However, stable transfection of the CSB gene reconstituted this defect. Semiquantitative PCR showed increased levels of mtDNA deletions in CSB knockout mice *in vivo* compared to wild-type littermates in the brain but not in muscle, cartilage spleen or testis, correlating with the neurodegenerative changes found in aging animals. In aggregate, extending and corroborating previous findings, these results strengthen the role of CS proteins in the repair of oxidative damage to mtDNA and indicate a heterodimeric composition of both proteins as requirement for mitochondrial import. Furthermore, the correlation of neurodegenerative symptoms and defective removal of oxidatively induced mtDNA deletions in CS knockout mice indicates a close relationship of oxidative stress, its repair by CSA and CSB and processes such as neurodegeneration and aging.

281 [Oral 032]

Requirement of a Thymic Route for UV-Induced Tolerance

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We currently showed that UV-induced tolerance is mediated by the induction of a new type of T suppressor cells, called $CD4^+CD25^+$ T regulatory cells (Treg), which are recently highlighted to counteract the onset of various autoimmunity, including experimental autoimmune encephalomyelitis or type 1 diabetes. Treg, in general, ultimately require to be maturated in thymus, leading to a concept of central tolerance. Although the involvement of Treg in UV-induced tolerance is no doubt, it is still uncertain how and where UV-induced Treg are generated. To address this issue, we generated athymic C3H mice, which were thymectomized at an age of day 3 and grown to 8–10 wk. Using athymic mice we tested the involvement of thymus in contact hypersensitivity (CHS) and its modulation by UV. Athymic mice showed vigorous CHS response, indicating that the induction of T effector cells (Tef) was not impaired. However, UV-induced tolerance was not observed in athymic mice, suggesting requirement of thymus in this process. Even when T cells obtained from naive animals were transferred intravenously to athymic mice, UV-radiation failed to induce tolerance, further confirming the significant role of thymus in this process. Upon transplantation of age-matched thymus into kidney capsules of adult athymic mice, they regained the ability to undergo UV-induced tolerance. Collectively, the present study demonstrates the involvement of thymus in UV-induced tolerance and thereby suggests that UV-induced tolerance is acquired central (thymic) tolerance.

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UV Induced Hepatocyte Growth Factor from Dermal Fibroblasts Protects Keratinocytes and Fibroblasts from UV-Induced Apoptosis

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Hepatocyte growth factor (HGF), a multifunctional cytokine produced primarily by cells of mesenchymal origin, is able to prevent UVB induced apoptosis of human keratinocytes (KC). In the skin a continuous cross talk between dermal fibroblasts (FB) and epidermal KC is the prerequisite for tissue homeostasis in health and disease. Therefore we investigated the effects of cytokines released by KC after UV irradiation as well as the effects of direct UV irradiation on the HGF production by human FB.

We found that the supernatant of UVB irradiated KC strongly induced HGF production by FB and that this effects was mainly due to the presence of IL-1α. Like recombinant HGF, the supernatant of these stimulated FB protected KC from UVB induced apoptosis. That the protection was indeed due to HGF in the FB supernatant was shown by the fact that most of the anti-apoptotic activity was blocked by anti-HGF antibodies. Direct irradiation of FB with UVB had virtually no effect on the levels of HGF mRNA and secreted protein. In contrast UVA_1 irradiation led to a strong up-regulation of HGF mRNA within hours and secretion of the protein after 2 days. We hypothesized that the lag time for HGF secretion as compared to mRNA induction was due to a auto-consumption of HGF by FB. Indeed when we added a neutralizing anti-HGF antibody to the fibroblasts cultures after UVA_1 irradiation we observed a dramatic rise of FB apoptosis.

In conclusion we have shown for the first time that IL-1α released by KC after UVB irradiation as well as direct UVA_1 irradiation induce human FB to secrete HGF, which in an autokrine and parakrine fashion acts anti-apoptotic for FB and KC respectively.

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UVA-Induced Oxidative Damage and Cytotoxicity Depend on the Mode of Exposure

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The reciprocity rule (Bunsen-Roscoe law) states that a photochemical reaction is directly proportional to the total energy dose, irrespective of the dose distribution. This law is taken for granted in photomedicine, although the influence of irradiation intensity and dose distribution is largely unknown. We have examined in a tissue culture model the effects of fractionated versus single dose UV exposure from a metal halide source on cell death, DNA synthesis, glutathione and membrane damage. Exposure to fractionated UVA was followed by an increase of cell death compared to the single dose, when intervals between the fractions were short (10 to 60 minutes). Long intervals (>120 minutes) had the opposite effect. Corresponding results were obtained for DNA synthesis. The increased cytotoxicity of dose fractionation with short intervals could not be abrogated by non enzymatic antioxidants (astaxanthin, ascorbic acid, α-tocopherol). Fractionated irradiation with short intervals led to a higher depletion of glutathione and to enhanced formation of TBARS compared to an identical single dose. Long intervals between the fractions induced the opposite effect. Taken together, these data indicate that the oxidative effect of UVA and the antioxidative capacity of its cellular targets depend on the mode of exposure.

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Influence of MC1R, GSTT1 and GSTM1 genotypes on UV sensitivity

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Variations in the melanocortin-1 receptor gene (MC1R) and in the glutathion-S transferase genes mu1 (GSTM1) and theta 1 (GSTT1) are thought to influence UV-sensitivity at different levels. MC1R is one of the major genes that determine skin pigmentation because the melanocortin-1 receptor regulates eumelanin synthesis. GSTT1 and GSTM1 are expressed in the skin and detoxify products of oxidative stress reactions occurring in response to UV-irradiation. The possible influence of interactions of genetic MC1R and GST variants have not been thoroughly investigated yet.

We determined the minimal erythema dose (MED) of ultraviolet B (UVB) irradiation as well as the immediate pigmentation dose (IPD) and the delayed pigmentation dose (DPD) of ultraviolet A_1 (UVA_1) irradiation in 93 healthy volunteers. Genetic variations of the coding region of the MC1R gene were determined by direct cycle sequencing. GSTM1 and GSTT1 null genotypes were analyzed by multiplex PCR.

Six frequent and six rare variants of the *MC1R* gene were detected (allele frequency >6% and <1.5%, respectively). The R151C and R160W polymorphisms were associated with red hair, fair skin type and nevus count. R151C was also associated with a reported history of severe sunburns before the fifteenth birthday. The R151C and R160W polymorphisms were associated with DPD and IPD of UVA_1, but this association was related to the association of these polymorphisms with red hair and fair skin. Independent associations were found between the V60L polymorphism and the MED of UVB ($p = 0.0145$), and between the V92M polymorphism and the DPD of UVA_1 ($p = 0.0174$). In contrast to earlier investigations, there was no association between GSTT1 and M1 genotypes and UVB sensitivity. Lack of GSTT1 was associated with a higher IPD of UVA_1. Our results confirm complex interactions between MC1R variants and UV sensitivity that are partially independent of phenotypic characteristics. There was no evidence for a significant interaction of MC1R with GST variants in UV sensitivity.

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Withdrawn

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From Melanogenesis to Melanoma: Modulation by Telomere Homologous Oligonucleotides (T-oligos)

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Pigmentation is a highly regulated process involving differentiation of melanocytes and pigment production. Tanning, as it occurs after UV-exposure is part of a finely orchestrated DNA-damage response. The exact molecular mechanisms and sensors involved in mediation between cellular repair and pigmentation, however, remain to be elucidated.

We have shown that small DNA fragments homologous to the 3'overhang of telomeres are capable of inducing pigmentation in the absence of DNA damage both *in vitro* and *in vivo*. This response is regulated by p53.

Also, highly metastatic human melanoma cells (MV3, G361 and UISO Mel6) cells were treated with T-oligos (20uM). In all 3 cell lines apoptosis could be induced within 48 hours as demonstrated by TUNEL assay and subsequent FACS analysis. Results revealed an increase in apoptosis between 50% and 70% compared to diluent treated controls, depending on cell line. In detail, G361 cells revealed the strongest induction of apoptosis (69%) after T-oligo treatment, compared to 57% in MV3 cells and 46% in UISO-Mel6 melanoma cells.

Although the exact mechanisms and downstream sensors by which T-oligos regulate cellular differentiation and apoptosis are poorly characterized, common downstream regulators, such as p53, p21 and the E2F transcription factor are involved. Potentially differentiation as well as apoptosis as they occur after DNA damage and also after disruption of the telomere loop structure may be an evolutionary preserved cancer prevention mechanism. T-oligos might mimic these responses in the absence of DNA damage.

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Repression of Nuclear Hormone Receptors, their Target Genes and Co-Regulatory Proteins by UVB Irradiation in Keratinocytes

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In the present study we evaluated whether ultraviolet (UV)B irradiation alters the expression of nuclear hormone receptors and their ability to transactivate response elements in their target genes. mRNA levels of PPAR-alpha and LXR-alpha were decreased by 50% 24 hours after UVB treatment of primary cultured keratinocytes. As previously reported retinoid X receptor (RXR)-alpha and vitamin D receptor (VDR) were also decreased 24 hours after UVB exposure. In contrast PPAR-delta was increased, and LXR-beta, CAR, and TRs were unchanged. In these experiments PPAR-gamma was not detectable on Northern blots in cultured keratinocytes. Time course studies revealed that the mRNA repression occurred as early as 6 hours after UVB exposure. In addition, mRNA levels of acetyl-CoA-oxidase, a target gene for PPAR-alpha, and ATP-binding cassette transporter (ABC)-1, a target gene for LXR-alpha, were similarly decreased after UVB exposure. Furthermore, cultured keratinocytes transfected with a PPAR or LXR response element showed a marked decrease in promoter transactivation following UV-B irradiation. This decrease was not restored by either ligand treatment, over-expression of the receptor, or the combination of the two. Finally, we also observed down regulation of the co-regulatory protein DRIP, suggesting that the inhibition of gene expression by UV light is secondary to not only decreases in PPAR and LXR alpha but also to post receptor mechanisms that link the binding of transcription factors to DNA with increased transcription. The UV-induced down regulation of PPAR-alpha, LXR-alpha and DRIP results in decreased expression of downstream target genes that could reduce the movement of cholesterol out of cells and the oxidation of fatty acids thereby providing UV injured cells with lipids for the synthesis of new membranes.

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Activation of P38 Map Kinase by Ultraviolet Irradiation Limits Early Activation of NF-κB, Which is Required for Induction of Primary Cytokines IL-1β and TNF-α in Human Keratinocytes

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Primary pro-inflammatory cytokines IL-1β and TNF-α activate transcription factor NF-κB, which in turn stimulates expression of IL-1β and TNF-α. This positive feedback loop is limited by p38 MAP kinase, via phosphorylation of HSP27, which inhibits IκB kinase-β, thereby blocking phosphorylation and degradation of the NF-κB inhibitor IκB-α. UV irradiation induces IL-1β, TNF-α, and p38 MAP kinase in human skin *in vivo*. We have investigated the role of p38 MAP kinase in UV-activation of NF-κB in human skin and cultured human keratinocytes. We found that NF-κB activation, measured by nuclear localization, lags IL-1β and TNF-α induction by 2–4 hours, in human skin. Similarly, UV irradiation does not cause detectable NF-κB nuclear translocation prior to IL-1β or TNF-α induction, whereas treatment with IL-1β or TNF-α causes readily detectable nuclear translocation, in human keratinocytes. These data raise the question of whether UV activation of NF-κB is a consequence, or a cause, of IL-1β and TNF-α induction. To address this question, we utilized adenovirus-mediated expression of dominant negative inhibitors of NF-κB activators (IκB kinase-β, and IκB-α). Dominant negative IκB kinase-β or IκB-α completely prevented UV induction of IL-1β and TNF-α mRNA, indicating that the NF-κB pathway is required for UV induction of these cytokines. Sensitive ELISA-based DNA-binding assay revealed that UV irradiation rapidly induced a small (2-fold), but significant ($p < 0.05$) increase in DNA binding of p50/p65 NF-κB heterodimers, in human keratinocytes. UV irradiation also activated p38 MAP kinase within 30 minutes (3-fold, $p < 0.05$). Inhibition of p38 MAP kinase with SB203580 augmented UV-induced NF-κB DNA-binding 2-fold ($p < 0.05$). These data indicate that early activation of NF-κB is required for UV induction of IL-1β and TNF-α. This early UV activation of NF-κB pathway is limited by the actions of p38 MAP kinase, and difficult to detect by measurement of nuclear translocation. p38 MAP kinase functions to negatively regulate NF-κB, and thereby prevent excessive induction of IL-1β and TNF-α by UV irradiation in human skin.

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Phototherapy Inhibits Immediate Type Hypersensitivity Reaction in the Skin and Nasal Mucosa

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Since phototherapy has been shown to exert both local and systemic immunosuppressive effects, we investigated if it has also an influence on the immediate type hypersensitivity reaction in the skin and nasal mucosa. A significant inhibition (more than 35%) of wheal formation in skin prick test (SPT) was obtained only after irradiation with erythematous doses of UVB light. Although, UVA (0.5–2 J/cm^2) and visible (VIS) light (2–8 J/cm^2) had no effect on SPT, mixed irradiation with suberythematous doses of UVB (5%), low doses of UVA (25%) and VIS (70%) referred as mUV/VIS, resulted in significant (more than 55%) inhibition of wheal formation. To assess the effect of phototherapy on immediate type hypersensitivity in the nasal mucosa, we conducted a double-blind, placebo-controlled study in 49 patients with ragweed-induced allergic rhinitis. We performed intranasal irradiation 3 times a week for 3 weeks either with increasing doses of mUV/VIS (starting dose 1.7 J/cm^2) or with low intensity visible light (starting dose 0.06 J/cm^2) as placebo. Phototherapy significantly decreased scores for sneezing ($p = 0.016$), rhinorrhea ($p = 0.007$), nasal itching ($p = 0.014$) and total nasal score ($p = 0.004$), whereas no improvement was observed in the placebo group. In nasal lavage samples phototherapy resulted in a significant decrease of eosinophil cell counts ($p = 0.009$), ECP ($p = 0.028$) and IL-5 levels ($p = 0.047$). In the circulation, significantly higher serum IL-10 levels ($p = 0.002$) and IL10+CD4+T cells ($p = 0.017$) were found after phototherapy compared to that of placebo treatment. *In vitro* irradiation of T cells and eosinophils with mUV/VIS light induced dose-dependent apoptosis and inhibited the mediator release from RBL-2H3 basophils. Our results showed that phototherapy inhibits immediate hypersensitivity in the skin and nasal mucosa, acting at multiple point of the immunological process and therefore opens new opportunities for the treatment of immune-mediated skin and mucosal diseases.

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Effects of UV Irradiation on Cellular Responses and DNA Damage of Human Keratinocytes Harbouring HPV16

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In Epidermodysplasia verruciformis and some immunocompromised patients such as organ transplant recipients, cooperative effects of human papillomavirus (HPV) and ultraviolet (UV) radiation have been postulated in the development of non-melanoma skin cancers. The tumor suppressor protein p53 is a critical mediator of the cellular response to genotoxic agents by inducing either growth arrest, DNA repair or apoptosis. Transforming activity of high risk HPV is associated with loss of both cell cycle control and DNA repair systems. The aim of our study was to determine the effects of UV irradiation on cellular responses and DNA damage in HPV16 infected human keratinocytes. We used two keratinocyte lines, SKv-e and SKv-I, established from a bowenoid papulosis lesion and different in their viral genome integration, *in vitro* proliferative potential, and *in vivo* tumorigenicity in mice. Firstly, we showed that the weakly proliferative and tumorigenic SKv-e cell line was more sensitive to solar simulated radiation, more specifically to UV-induced apoptosis compared to the highly proliferative and tumorigenic SKv-I cell line. We demonstrated that SKv-e cell line accumulated strongly p53 and p21 proteins in their nucleus and that the p21 protein nuclear accumulation resulted from a p53-dependent transcriptional activation after UV irradiation. Moreover, we showed for the two SKv cell lines that UV irradiation induced the same level of CPD and (6–4) photoproducts whereas, for a same UV dose, in SKv-e cell line higher level of 8-oxodG was formed. Interestingly, this higher oxidative DNA damage formation in SKv-e cell line was related with low glutathione peroxidase activities and glutathione contents when compared to SKv-I cell line. Furthermore, CPD and (6–4) photoproducts are repaired to the same rate for the two cell lines although the repair of (6–4) photoproducts was most efficiently achieved compared to CPD. On the contrary, 8-oxodG was not repaired for the two cell lines. In conclusion, our results demonstrate that p53 remain inducible and functional in HPV16-infected cells and confirm a cooperative role of HPV and UV in the transformation of keratinocytes by allowing DNA damage accumulation in HPV infected cells.

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The Effect of UVA_1, UVB and SSR on p53 Activation, and p21 $^{Waf1/Cip1}$

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High dose UVA_1 therapy (doses in the order of 130 J/cm^2) has been used to effectively treat atopic dermatitis and scleroderma. UVA_1 has been shown to cause a dose dependent increase in P53 expression in keratinocytes, but its effect on the activation of p53 by phosphorylation, and on other downstream mediators of apoptosis such as P21 $^{Waf1/Cip1}$, has not been examined.

Five adult volunteers were exposed to geometric dose series of UVA_1 (10-100 J/cm^2 from a high output source (Dr Hönle, Dermalight ultra 1, irradiance 70-77mW/ cm^2)), TL-01 UVB (50-550 mJ/cm^2) and SSR (5.6-30 J/cm^2) irradiation on the non-exposed buttock skin and the minimal erythema dose (MED) for each was determined at 24 hours. Separate sites on the buttocks were subsequently irradiated with a 3 $\times$ MED dose of UVA_1, TL-01 and SSR. At 24hr 4mm punch biopsies were taken from each irradiated site and an adjacent unirradiated control site, fixed in paraffin and underwent immunohistochemical staining for p53 (Do-1), activation of p53 (assessed by phosphorylation at serine 15 & 392) and p21. Cell staining was expressed as the mean number of cells stained per 3 high power fields (HPF) and as a % of 1000 cells.

UVA_1 produced relatively little staining of p53 (mean cell count per HPF 16 (SD10)) and no evidence of p21 expression or p53 activation in contrast to TL-01 UVB (mean count per HPF (SD) of 147 (62) for Do-1, 54 (50) for serine 15 and 54 (31) for p21) or SSR irradiation (mean count per HPF (SD) of 137 (50) for Do-1, 35 (26) for serine 15 and 66 (28) for p21).

These data indicate that there are fundamental differences in the effects of UVA_1 on p53 and its activation markers, and may explain in part, the differential effect of these phototherapies.

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VEGF-A protects endothelial cells from UVB-induced cell death

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Skin alterations observed after single and prolonged exposures to UVB include erythema, dilation of dermal blood vessels, vascular hyperpermeability and epidermal hyperplasia. Vascular Endothelial Growth Factor (VEGF) is one of several pro-angiogenic factors that are induced in skin after UVB irradiation, and epidermal keratinocytes (KC) are the major source of VEGF in skin. Using the Cre/LoxP system under the control of the Keratin 5 promoter, we have generated mice in which VEGF has been inactivated in epidermal KC (VEGF-A$^{\Delta K5\text{-}cre/\Delta K5\text{-}cre}$) and used these animals to study the contribution of KC-derived VEGF to acute and chronic UVB-induced photo-damage.

We have shown previously that high dose UVB irradiation induced superficial wounds in mutant but not in control mice, and both after a single high dose and chronic low dose UVB irradiation blood vessel response was impaired in the mutant mice.

Several reports have implicated VEGF as a major survival factor for endothelial cells exposed to stress stimuli such as TNF-α, H202 and serum starvation. We found that exposure of HDMVEC to UVB irradiation in the presence or absence of VEGF dose- and time-dependently significantly protected the cells from death. This suggests that VEGF protects from cutaneous photo-damage by preventing UVB-induced cell death in EC, thereby allowing enhanced vascularization and KC survival.

Understanding more about the mechanisms contributing to this impaired blood vessel response in the mutant mice may be used to better understand the biologic role of skin blood vessels in the pathogensis of acute and chronic photodamage.

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Carboxyfullerenes Localize at the Intracellular Level and Prevents Caspase Cascade Induced by UVB in Human Keratinocytes

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Water-soluble derivatives of buckminsterfullerene (C60) are a unique class of compounds with potent antioxidant properties. We have shown previously that carboxyfullerenes (CF) protect human keratinocytes from ultraviolet (UV) light-induced apoptosis. The aim of the present study was to evaluate the molecular mechanism underlying this effect. First of all, we wanted to determine the subcellular localization of CF in human keratinocytes. To this purpose, we added CF to keratinocyte cultures, stained them with an anti-CF monoclonal antibody followed by incubation with a FITC-conjugated antimouse antibody, and examined the cells under a confocal laser microscopy. CF appear to localize at the intracellular level in the cytosol, close to the nucleous. Moreover, the addition of CF reduced the percentage of depolarized mitochondria, as shown by JC-1 probe, and prevented the release of cytochrome c induced by UVB irradiation. We also confirmed that CF protect human keratinocytes from UVB-induced apoptosis. We next wanted to evaluate the influence of CF on the caspase cascade induced by UVB. While UVB induced the cleavage of both initiator and effector caspases at 24 hrs, CF prevented the activation of caspase-8, -9, -10. In addition, CF up-regulated the group of inhibitor of apoptosis proteins (IAP), which in turn inhibit caspase activation. These results demonstrate that CF protect human keratinocytes from apoptosis through the modulation of caspase cascade. The antioxidant property, the prevention of mitochondrial depolarization and release of cytochrome c together with the intracellular location suggest that CF could indeed act at the mitochondrial level.

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Low-dose UVB Induces a p53-Dependent Gene Program that Increases the Resilience of Keratinocytes Against Upcoming UVB-Insults

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In previous work we have shown that pre-exposure to a low dose of UVB-damage triggers an adaptive response in keratinocytes, provided that the time-interval between consecutive UVB-insults is long enough (24 h). This adapative response is discerned by an increase in survival and a decrease of apoptosis. This study investigates the mechanism behind this adapative response.

One protein central to the UVB-response of human keratinocytes is p53. By transactivating genes involved in either cell cycle arrest or DNA repair, p53 has a leading role in the recovery from UVB-damage. The possible role of two of these p53-target genes, p21 and p53R2, in the adaptive response was examined. p21, an inhibitor of cyclin-dependent kinases, is an important mediator of the p53-induced G1/S arrest following UVB-damage. p53R2, a ribonucleotide reductase, facilitates DNA repair in response to UVB by creating a fresh supply of deoxyribonucleotides. We found that, while p53 levels increase in response to both low (12 mJ/cm^2) and high (32 mJ/cm^2) UVB-doses, only low levels of UVB damage result in upregulation of p21 and p53R2. Furthermore, we found that UVB-induced upregulation of these genes takes time, and peaks 24 hours after the UVB-insult.

Untreated keratinocytes, exposed to a high dose of UVB, never show elevated levels of either p21 or p53R2. By exception however, significantly increased basal levels of both p21 and p53R2 can be observed when these keratinocytes are first pre-exposed to a low dose of UVB (8 mJ/cm^2) 24 hours before the high-dose (32 mJ/cm^2) exposure. Monitoring of both cell survival and caspase-3 induced PARP cleavage confirms that these pre-exposed keratinocytes are more resistant to higher levels of UVB damage compared to untreated keratinocytes.

Similar results are found when a high dose (24 mJ/cm^2) is fractionated into three low doses of UVB (8 mJ/cm^2) with a set amount of time (30', 1 h, 3 h or 24 h) between subsequent UVB-insults. Elevated p21 and p53R2 levels are only observed with longer intervals (24 h) between subsequent UVB-insults. In parallel and thus consistent with the expression pattern of p21 and p53R2, stimulation of survival and decrease of apoptosis are also only observed with these longer intervals.

These results indicate that the adapative response to UVB-irradiation, induced by a pre-exposure with a low dose of UVB, is the result of a p53-dependent transcriptional activation of genes with an active role in growth arrest and DNA repair. However, the adaptive response only works optimal when the interval between consecutive UVB-doses is long enough (e.g. 24 hours), since upregulation of these p53-target genes requires time.

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Adaptive Response of Hairless Mouse Epidermis to Maintain Optimal Vitamin A Concentration During Long Term UV Exposure

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The depletion of cutaneous vitamin A by a single exposure to UVB is well documented in hairless mice. When a suberythemal UVB dose is received three times per week, tumours develop after about 15 weeks. In this paradigm simulating long term UV, UVB act both as an initiator and a promoter of skin cancer. In this study, we wondered whether the depletion of cutaneous vitamin A was maintained during long term UVB exposure. Hairless mice were exposed to a suberythemal UVB dose of 70 mJ/cm^2 3 times per week for one, 5, 10 or 20 weeks. Skin tumours began to appear in the last group after 14 week, all mice beared tumours after 18 weeks, and had an average number of 16 tumours larger than 1 mm per mouse after 20 weeks. Vitamin A (retinol and its esters) and vitamin E (α-tocopherol) were assayed by HPLC in the epidermis, the dermis, the plasma and the liver. A piece of skin was fixed in formol and used for histological slices (H/E staining). In the UVB irradiated mice, epidermal vitamin A concentration (basal value in non irradiated controls: 1.58 $\pm$ 0.12 nmol/g) decreased dramatically during the first week of UVB irradiation to 12% of that of non irradiated controls, whereas after 5 weeks of irradiation it was similar to that of non irradiated controls, and 180% of that of controls after 20 weeks of UVB irradiation. On the other hand, dermal vitamin A (0.75 $\pm$ 0.1 nmol/g) did not change significantly during the whole paradigm. After 10 weeks of irradiation, there was no significant change in plasma (3.06 $\pm$ 0.36 μM) or liver (10.9 $\pm$ 2.3 μmol/g) vitamin A. Epidermal vitamin E (37.8 $\pm$ 1.2 nmol/g) was slightly increased after one week UVB (55.5 $\pm$ 3.5 nmol/g), but the difference between irradiated and non irradiated mice decreased as a function of the duration of UVB irradiation. In summary, there was a transient depletion of epidermal vitamin A at the beginning of the UVB irradiation paradigm, then epidermal vitamin A levels were similar or higher to those of non irradiated controls, especially at the time of cutaneous tumour development, whereas epidermal vitamin E was never depleted during the whole paradigm. The adaptive response of the epidermis to control vitamin A concentration during long term UVB irradiation suggests that epidermal vitamin A could play a role in counteracting the deleterious action of long term UVB exposure.

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***Cis*-urocanic Acid Induces NFκB Activation in Primary Human Keratinocytes**

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Ultraviolet radiation (UVR)-induced suppression of cutaneous cell-mediated immunity plays an important role in the development of photocarcinogenesis and susceptibility to infectious diseases.

Photoisomerisation of urocanic acid (UCA) from its *trans*- to *cis*- isomer is a significant event in the initiation of UVR-induced immunosuppression. The mechanisms by which *cis*-UCA modulates immune responses are not yet fully defined. However, the expression of many genes involved in immunological and inflammatory responses are under the control of the transcription factor NFκB, which is strongly upregulated by UVR. We therefore investigated the effect of UCA isomers on the activation of NFκB in order to determine a possible involvement for this transcription factor in *cis*-UCA mediated immunosuppression.

Human primary keratinocyte cultures were established from biopsies of non-exposed buttock skin from healthy white-skinned volunteers (n = 16). Keratinocytes were incubated in 100 μg/ml of *cis*- or *trans*-UCA in PBS. Nuclear extracts were performed after 2 h and 24 h and analysed by electrophoretic mobility shift assay. *Cis*- but not *trans*-UCA stimulated the release of NF-κB to the nucleus at both time-points and was greatest at 24 h ($p = 0.0065$).

The induction of immunosuppression by *cis*-UCA may involve the activation of the NFκB–pathway in keratinocytes.

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Assessment of 4 Xeroderma pigmentosum Group C and G Gene Polymorphisms and Risk of Cutaneous Malignant Melanoma: A Case-Control Study

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Individuals with the rare nucleotide excision repair deficiency syndrome xeroderma pigmento-sum (XP) are sun-sensitive and exhibit a 1000-fold increased risk for developing skin cancers including malignant melanoma. Inherited polymorphisms of XP genes may, thus, contribute to subtle variations in DNA repair capacity and genetic susceptibility to melanoma. We investigated the role of three variant alleles of the DNA repair gene XPC and one variant allele of the XPG gene in a hospital-based case-control study of 294 Caucasian patients from Germany with malignant melanoma and 375 healthy control individuals from the same area matched by sex. The polymorphisms G1580A (XPC exon 8; Arg492His), T1601C (XPC exon 8; Val499Ala), G2166A (XPC exon 10; Arg687Arg), and C3507G (XPG exon 15; Asp1104His) were not in linkage disequilibrium. The allele frequencies (cases : controls) were for 1580A 6.29% : 5.63%, for 1601C 79.08% : 78.28%, for 2166A 26.19% : 28.13%, and for 3507C 20.14% : 21.39%. No significant deviation of the observed genotype distributions from the expected genotype distributions as predicted by the Hardy-Weinberg theory were detected. We found no association of the homozygous 1580A, 1601C, 2166A, and 3507G genotypes with increased risks of melanoma: OR 1.254 (95%-CI: 0.486–3.217), OR 1.108 (95%-CI: 0.629–1.960), OR 0.817 (95%-CI: 0.490–1.358), and OR 1.168 (95%-CI: 0.670–2.044), respectively. Exploratory analyses of subgroups indicated no association of these genotypes with increased risks for the development of multiple primary melanomas ($n = 28$), a negative family history for melanoma ($n = 277$), melanomas in individuals with a low number of nevi ($n = 273$), melanomas in individuals older than 55 years ($n = 142$), and melanomas thicker than 1mm ($n = 126$). Our results do not support the hypothesis that the four polymorphisms in the XPC and XPG genes affect susceptibility to melanoma.

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Relationship Between Skin Typology and UV Sensitivity: Photoprotection Due to Constitutive Pigmentation

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Sun exposures are responsible for deleterious effects ranging from sunburn, to photoaging and skin cancer. These consequences are likely to be influenced by constitutive pigmentation, as demonstrated by i) epidemiological studies on skin cancer incidence in Caucasian and African American population ii) relationship between the MED (Minimal Erythemal Dose) value and skin typology. The latter is commonly determined according to Fitzpatrick's classification based on erythema and tanning ability. Nevertheless this classification has some limits in terms of quantification, *ex vivo* conditions and relevance for Asian skin for example. Except for erythemal reaction, only few experimental data are available on the relationship between UV sensitivity and skin typology. We analyzed 31 *ex vivo* skin samples objectively classified in 6 groups (from very light skin to dark skin) according to their values of Individual Typology Angle (ITA) based on colorimetric parameters. The BED (Biologically Efficient Dose) was determined for each sample by quantifying the sunburn cells after UV exposure (UVR solar simulation, UVB + UVA). Several typical biological markers related to UV response, other than erythema, were analyzed such as DNA damages, apoptosis, and p53 accumulation. We found a statistically significant correlation between ITA and BED and between ITA and DNA damages. Interestingly, DNA damages were distributed throughout the whole epidermal layers in light skin while they were absent in the basal layer of dark skin. Our data support, at the cellular level, the relationship between UV sensitivity and skin typology. They emphasize the impact of DNA damage accumulation in the basal in relation with the prevalence of skin cancer. Moreover typological delimitations for skin sensitivity to UV could be envisaged based on the ITA value.

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Genomic Scale Analysis of *in vivo* UVB-Irradiated Human Epidermis

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Background. Several recent studies have employed genomic scale analysis to study UVB regulated gene expression in human skin. These studies are mostly based on UV irradiated, cultured cells that differ substantially from the intact tissues they are supposed to imitate. The purpose of the present study was to analyze the differential expression of UVB regulated genes in intact human epidermis following *in vivo* UV irradiation.Methods:

The forearms of human volunteers were exposed to 4 MED of UVB *in vivo*, followed by removal of suction blister roofs from exposed and non-exposed epidermis 2 h, 24 h, and 72 h after irradiation. RNA samples were analyzed using oligonucleotide microarray (Affymetrix) technology analyzing over 8,500 verified human genes from the NCBI RefSeq database simultaneously. Verification of selected genes was performed by semi-quantitative RT-PCR.
Results. 615 common active genes were changed at least 2 fold in at least one time point in all experiments. Classification of these genes into functional categories revealed that multiple, biological processes are globally affected by UVB. The differentially regulated genes included genes involved in signal transduction, cell proliferation and differentiation, cell signaling, DNA repair, and apoptosis. Cluster analysis revealed that the majority of the UVB regulated genes were found 24 hours after irradiation, suggesting that a single, low UVB exposure of human epidermis results in only transient expressional changes.
Conclusion. Our results illustrate the power of global gene expression analysis of human epidermis to identify molecular pathways involved in UV induced photodamage.

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Melanocortin 1 Receptor Genotype and Cutaneous Responses to a Single Dose Of Ultraviolet Radiation

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Genetic variants of the melanocortin 1 receptor (MC1R) are associated with red hair, fair skin and an increased susceptibility to skin cancer. This increased risk may result from both pigmentary and non pigmentary effects of the variant receptor, however the exact mechanism is unclear. In this study we have investigated whether *MC1R* variants alters the cutaneous responses of human skin to a single dose of ultraviolet radiation (UVR).

Thirty subjects were recruited from patients due to undergo UVB therapy for psoriasis. Untanned buttock skin was exposed to incremental doses of UV ($\sqrt{3}$) from a TL12 lamp. After 24 hours, UV induced erythema and basal melanin index readings were obtained using an erythema-melanin index meter, and 6 mm biopsies were taken from unirradiated skin and from skin exposed to 200 mJ/cm^2 UV. The amount of DNA damage and cutaneous responses to UV were assessed by immunostaining for cyclobutane pyrimidine dimers (CPD) and p53 respectively, and quantified by image analysis. Melanin content was assessed by Masson-Fontana stain and expression of MC1 receptor within the skin determined by immunohistochemistry. Subjects were genotyped at *MC1R* and designated as wild type (WT), single variant allele, and two variant alleles at this locus; alleles containing *MC1R* variants which are not thought to impair receptor function were designated as pseudo-wild type (pWT).

Twenty seven of 30 subjects were WT *MC1R* (14 individuals) or had a single variant *MC1R* allele (13 individuals), and skin biopsies were obtained from 13 WT and 12 single variant subjects. Basal melanin index, melanin content and expression of MC1R did not differ significantly between subjects with WT and a single variant allele. At 24 hours there was no significant difference in UV-induced erythema nor in the percentage of CPD positive epidermal cells or p53 positive epidermal cells between subjects with WT and a single *MC1R* variant.

The results suggest that there is no significant difference in the cutaneous responses to a single dose of UVR between individuals with WT *MC1R* and subjects with a single variant *MC1R* allele.

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The Effect of Photochemotherapy (PUVA) on Dermatoscopic Features of Melanocytic Nevi

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Photochemotherapy with psoralen and UVA (PUVA) is a very effective treatment for patients with psoriasis. In the light of an increased long-term risk for the development of skin tumors such as squamous and basal cell carcinoma, and as recently reported also for melanoma, we studied the effects of PUVA therapy on dermatoscopic features of melanocytic nevi.

During an 8-weeks course of PUVA therapy a total of 103 nevi in 6 patients were followed and dermatoscopic photographs were taken before and at the end of PUVA therapy. Follow-up dermatoscopic photographs were taken 3 months after the last PUVA treatment. Two blinded investigators compared the three images of each melanocytic nevus without knowing whether the photographs were taken before or at the end of PUVA, or 3 months after PUVA therapy. The evaluation of the dermatoscopic images comprised the following dermatoscopic features: Total pigmentation, area, globules/dots, faded pigment network, asymmetry, various colours, regression structures, and atypical pigment network. In a first step of evaluation the presence of the particular features was determined. Then, the expression of the respective features in the three images was compared and defined as equal, more, or less. For nevi showing an increased expression of one of the features at the end of PUVA, it was determined whether this feature was still present 3 months after PUVA therapy.

At the end of PUVA, in 103 nevi total pigmentation was increased in 14%, decreased in 15%, and was unchanged in 71%. Area increased in 22%, decreased in 11%, and was unchanged in 67%. Of the other features, globules/dots (in 70 of 103) and faded pigment network (in 60 of 103) were most frequently increased (i.e., 43% and 45%, respectively). In 13 (13%) nevi the expression of at least one feature suspicious for malignant transformation such as asymmetry, various colours, regression structures, and atypical pigment network, was increased at the end of PUVA. Three months after the end of PUVA in 10 nevi at least one of these dermatoscopic feature was still present. Only in 4 nevi the expression of two or more features suspicious for malignant transformation were increased at the end of PUVA, and persisted throughout a three months-period after the end of PUVA therapy.

In conclusion, PUVA therapy is capable of altering dermatoscopic features of melanocytic nevi. In a small number of nevi features suspicious for malignant transformation develop during the course of PUVA and may persist for months after PUVA therapy.

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Apoptosis and Proliferation of Human Epidermal Keratinocytes After an Acute UV Exposure: An *in vivo* Immunohistological Study

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Ultraviolet radiations (UVR) participate to skin carcinogenesis and photoaging. When epidermis, the outermost part of the skin, is exposed to UVR, damaged keratinocytes can undergo apoptosis, a programmed cell death that removes them from the tissue and contributes to its protection and maintenance. Therefore the question related to a decrease of such a phenomenon in human skin during aging raises. The aim of this study was to investigate apoptosis in normal sun-exposed skin following an acute UV radiation depending on the age. Apoptosis was assessed in the arm of young ($n = 12$, mean aged 25.5) and aged female volonteers ($n = 12$, mean aged 67.1) before and 24 h after sun exposure to a 3-fold minimal erythema dose (MED). Immunohistological study was carried out on 10 μm cryosections obtained from 3 mm skin biopsies. Apoptosis together with proliferation was investigated using antibodies against active caspase-3 and Ki-67 respectively. Moreover, β_1 integrin and active caspase-3 double staining were performed in order to determine localisation of apoptotic cells. These results indicate that proliferation index (PI) is about two times greater in young than in aged epidermis. However, after the acute sun exposure, while proliferation is greatly inhibited in young epidermis (PI from 216 to 68), it slightly decreased in aged epidermis (PI from 115 to 85). No spontaneous apoptosis in young and aged volunteers was observed without any acute UV exposure. 24 hours following sun exposure, apoptosis was strongly induced in both groups with a greater sensitivity of the young volunteers as opposed to aged ones. Double staining with β_1 integrin indicated that percentage of caspase-3 active cells in basal cell compartment is greater in aged volunteers than in younger. Taken together, these results indicate that an acute UV exposure to 3 MED induced apoptosis more strongly in young than in aged epidermis and that this greater sensitivity of young epidermis is associated with an arrest of basal cell proliferation.

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Microarray Analysis of Chronically Sun-Damaged Human Skin

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Intrinsic, environmental, and lifestyle factors all contribute to the process of skin aging. Photoaging, a remodeling of the dermis that arises as a result of repeated exposure of skin to UV-light has been identified as the predominant contributing factor to the prematurely aged appearance of sun-exposed skin.

To study the phenotypic changes in human skin associated with chronic sun exposure at the transcription level, we had previously undertaken a Serial Analysis of Gene Expression (SAGE) of sun-damaged and sun-protected skin from a single patient undergoing elective facial plasty. We detected 34 genes with altered mRNA levels between the two full-thickness skin samples. Prior to a detailed study of the functional relevance to photoaging of some of these genes, it was critical to determine the inter-individual differences in gene expression profiles. We have used RNA preparations from skin obtained from 11 Caucasian patients with moderate sun-damage and analyzed the changes in mRNA levels encoded by more than 22,000 different genes represented on the Affymetrix U133A chip. We developed an algorithm that exploits dependencies between control and test chips by computing a paired t-statistic for each gene as a measure of differential expression and a false discovery rate for lists of genes called significantly changed based on permutation testing. Using this algorithm, we were able to identify 566 mRNAs with consistently altered steady-state levels as a consequence of chronic sun exposure. Here we also demonstrate that for one of the genes, a consistent change in the protein expression level was detected. The protein was localized to the epidermis by Immunohistochemistry (IHC) and the same trends that were seen in the Western blot analysis were maintained in IHC.

In conclusion, in this study, we were able to expand the number of genes assessed to more than 22,000. Furthermore, these results support the changes that were previously reported using SAGE for an individual patient and are reflective of a larger population of chronically photodamaged individuals.

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Heat Shock Inhibits UVA-Induced Cell Death

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The ability of heat shock to inhibit UVB-induced cell death is well documented in human keratinocytes. In the present study we investigated the influence of heat pretreatment on UVA-induced cell death. The human squamous carcinoma cell line A431 and the human fibrosarcoma cell line HT1080 were exposed to heat shock (3 h at 42° C) and subsequently to UVA from a metal halide source (315–390 nm, 40–113 J/cm^2, Mutzhas Supersun 5000, Germany). Viability, lipidperoxidation and 8-oxoguanine were determined after UVA-exposure by an MTT assay, TBARS measurement, and immunocytochemistry (OxyDNA, Biotrin), respectively. At 24 h after UVA-exposure the survival rate was markedly increased in heat pretreated A431 compared to controls at all dosages. This effect was highest when heat shock was applied 2 h before irradiation, and decreased continuously with increasing intervals. In contrast, heat shock applied 2 h before irradiation had no effect on TBARS and formation of 8-oxoguanine. Heat pretreatment in HT1080 showed no effect.

These results for the first time demonstrate that heat shock is able to inhibit UVA-induced cell death. The observed time course indicates that heat shock proteins might be related to this protective effect, which seems to be tissue specific. Initial data suggest that the heat related increase of survival occures independently of lipid peroxidation and oxidative DNA-damage.

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Experimental Study of Photo-Protection of Hydroxychloroqine and TCMs on Human Keratinocytes Damaged from Ultraviolet-B Irradiation

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Objective. To evaluate photo-protective efficiency of hydroxychloroqine and traditional Chinese medicines (EGCG, szechwan lovge rhizome and baikal skullcap root) on human keratinocytes (HaCaT cells) damaged from UVB irradiation and their mechanisms.Methods:

1. HaCaT cells were cultured in RMPI-1640 media with 10% fetal bovine serum and plated in 3.5 cm dishs with a equal amount (10^6).
2. Subconfluent HaCaT cells were shammed or irradiated with different dosages of UVB irradiation 30, 60, 90 mJ/cm^2 and treated with above TCM agents and (or) hydroxychloroqine.
3. Flow cytometric analysis was performed and cells in the G_1, S, and G_2/M phase of the cell cycle and apoptotic rate were determined with flow cytometery.
4. The mRNA expression levels of p53, p21, c-fos and GADPH gene were evaluated by reverse transcription-polymerase chain reaction (RT-PCR), the scanned intensity of each gene band to the corresponding band of GAPDH PCR product and normalized to the control sample.

Results. 1. UVB-induced apoptosis of HaCaT cells were dose-dependent, from 0.21% (0 mJ/cm^2) to 71.18% (90 mJ/cm^2); HCQ and TCMs showed protective effect on UVB-induced apoptosis, the apoptotic rate was reduced in all irradiating dosage levels.

2. UVB-irradiation caused S-phase arrest with an increase amount of cells in S-phase in 30 mJ/cm^2 level; but with increase of irradiated dosage, the amount of cells in S-phase descended rapidly. The intervention of the tested drugs could inhibit S-phase arrest induced by UVB.

3. UVB irradiation could induce mRNA expression of p53, p21 and c-fos in cultured HaCaT cells; and mRNA levels of p53, p21 and c-fos were decreased after TCM intervention in UVB irradiation group.

Conclusions. The cell cycle arrest and apoptosis induced by UVB irradiation on HaCaT cells could be inhibited with the intervention of HCQ and TCMs. The photo-protective mechanisms of above medicines may be partly related to inhibition of the expression changes of p53, p21, c-fos gene.

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The Effectiveness of PUVA Treatment in Severe Psoriasis is Impressively Increased by Addition of Several UVB 308 nm Excimer Laser Sessions

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Objectives. Both UV-light therapies of psoriasis, the one using psoralen and UV A (PUVA) and the other using narrow-band UVB 311 nm are one of the most successful treatments inducing clearance of psoriatic lesions. Unfortunately, both therapies need a number of more than 25 treatment sessions to clear up psoriasis and UV dosages depends on the MED testing in not involved skin. In contrast, excimer laser derived 308 nm UVB radiation targets only the involved skin, less than 12 treatments are necessary and the UVB dosages can exactly applied in the psoriatic lesions starting with the MED-I dosage. Although the UVB laser treatment were performed only in moderate psoriasis with an involved skin $<$20%, we were interested to combine UVB 308 nm excimer radiation and PUVA in severe psoriasis.

Methods. To analyze if the combination of the two phototherapies are more effective than PUVA alone, a prospective study was performed: 256 patients (170 men and 105 women, mean age 50 years) with severe psoriasis where included in the study. 113 patients were treated with PUVA, during the second group with 143 patient were treated with PUVA and several sessions of 308 nm excimer Laser. Clinical improvement and relapse was documented by PASI over a time period of 4 months.

Results. In the PUVA treated group there was a clearance of 67,3% (76/113), 23,0% (26/113) partial clearance and 9,7% (11/113) non-responder. 26 $\pm$ 7 treatments were necessary to get a complete clearance. The cumulative dose of UV A was 53,2 J/cm^2 $\pm$ 26,3 J/cm^2 and the average time of treatment was 6,5 weeks.

In the PUVA and excimer laser treated group, the rate of clearance with 63,6% (91/143), partial clearance 28,0% (40/143) and non-responders 8,4% (12/143) were similar. But the number of treatments in the clearance group was much lower (15 $\pm$ 6) and the cumulative UVA dose decreased to 22.9 J/cm^2 $\pm$ 5,8 J/cm^2 and the average time was 4,2 weeks. The number of the additionally applied treatments with Excimer Laser was 3,8 $\pm$ 1,9 with a dose of 4070 mJ $\pm$ 3293 mJ.

Conclusion: Clearance of psoriatic lesions can be achieved dramatically, very fast and with 57% reduction of UVA dosages if PUVA-treatment is combined with several sessions of 308 nm laser derived UVB light.

307 [Oral 042]

The First Cutaneous T-Cell Lymphoma (CTCL)-Associated Gene Aberration

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Patients with cutaneous T-cell lymphoma (CTCL) show a large variety of chromosomal aberrations with no previously known common or specific aberration. We have now identified a common aberration detectable in the majority of CTCL cases and the gene involved. Multicolour fluorescent *in situ* hybridization (multicolour FISH) was used to identify acquired chromosomal aberrations in 12 patients with mycosis fungoides (MF) or Sezary syndrome (SS), the most common forms of primary cutaneous T-cell lymphoma (CTCL). The most frequently affected chromosome was 12, which showed clonal deletions or translocations with a break point in 12q21 or 12q22 in 5 of 7 SS patients and clonal monosomy in the sixth patient. The distal and proximal breakpoints of two deletions were fine mapped to 12q21, and the breakpoint of a balanced translocation t(12;18)(q21;q21.2), was found in the minimal common region of these two deletions. The breakpoint was further fine-mapped by locus-specific FISH, and was found to disrupt a single gene. This gene, neuron navigator 3 (NAV3), also named POMFIL1, a newly described human homologue of *unc-53* in *C. elegans*, is located in 12q21.1. A missense mutation in the remaining NAV3 allele was found in one of two SS cases studied with a deletion. With locus-specific FISH, we have demonstrated NAV3 deletions in touch samples of the skin lesions of 4 of 8 (50%) patients with early MF (stages IA-IIA) and a deletion or a translocation in the skin or lymph node in 11 of 13 (85%) patients with advanced MF or SS. Thus, NAV3 is a novel putative target tumour suppressor gene, disrupted in most cases of as well early as advanced MF and SS patients. The demonstration of NAV3 deletion in clinical samples may provide a new, specific diagnostic test for CTCL.

308 [Oral 033]

Conditional Deletion of VEGF Impairs SOS-Dependent Skin Tumour Development in Transgenic Mice

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The vascular endothelial growth factor (VEGF) is a key mediator for normal and abnormal angiogenesis and is implicated in the development of many epidermal tumours. K5-SOS transgenic mice expressing a constitutively active form of the Ras activator Son of Sevenless (SOS) in the basal layers of the epidermis develop skin papillomas at 100% penetrance in a wild-type epidermal growth factor receptor (EGFR) background, whereas in a hypomorphic (wa2) EGFR background tumour development is severely impaired and can be induced by wounding.

To address the role of VEGF during skin tumourigenesis, we generated K5-SOS transgenic mice carrying a conditional (floxed) VEGF allele in an EGFR wild-type (EGFRwa2/+) and hypomorphic (EGFRwa2/wa2) background. Our studies show that deletion of VEGF in basal keratinocytes with a K5-Cre transgenic line severely impairs SOS-dependent skin tumour development. Whereas all EGFRwa2/+ K5-SOS mice develop tumours by 7 weeks of age, 80% of EGFRwa2/+ K5-SOS mice lacking VEGF in the epidermis (VEGF$^{\Delta ep}$) were still tumour free at this age. Although by 12 weeks, all EGFRwa2/+ K5-SOS VEGF$^{\Delta ep}$ mice had developed tumours, their average tumour volume was significantly smaller than in EGFRwa2/+ K5-SOS mice. The number of blood vessels present in EGFRwa2/+ K5-SOS VEGF$^{\Delta ep}$ skin tumours was significantly reduced suggesting that the delay in SOS-dependent skin tumour development is due to impaired angiogenesis. Surprisingly, in a hypomorphic EGFR background the lack of VEGF expression in keratinocytes completely inhibited SOS-dependent skin tumour development. Even after wounding, EGFRwa2/wa2 K5-SOS VEGF$^{\Delta ep}$ mice remained tumour free for at least 9 months. Interestingly, mice lacking only one VEGF allele started to develop tumours after wounding at the same time as EGFRwa2/wa2 K5-SOS mice, but their tumour volume was significantly smaller. These results show that VEGF deletion in the epidermis severely impairs SOS-dependent tumour growth in a wild-type EGFR background and completely inhibits tumour development in a mutant EGFR background even after wounding. The molecular and cellular mechanisms responsible for these phenotypes will be presented.

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Targeting Melanoma Cells and Melanomas in a Nude Mouse Model by Proapoptotic Genes

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Apoptosis resistance is a critical feature of tumor cells and may often be the cause for therapy resistance. The CD95/Fas signalling system is of general importance for the regulation of apoptosis as are pro- and antiapoptotic Bcl-2-related proteins which control the release of mitochondrial proapoptotic factors at the level of the mitochondrial membrane.

In melanoma cell lines, expression of the receptor (CD95) but not of the ligand (CD95L) was found at the mRNA and at the protein level. Overexpression of CD95 did not trigger apoptosis by itself but enhanced sensitivity to agonistic receptor activation, after transient overexpression. On the other hand, melanoma cells responded with increased apoptosis both after transient and after stable transfection and expression of CD95L in a tetracycline-inducible expression system. Activation of the downstream signalling cascade (cleavage of caspase-8, Bid and caspase-3 as well as release of cytochrome c) was confirmed. In nude mice CD95L-transfected melanoma cells lost tumorigenicity when CD95L expression was induced, and partial regression of pre-existing melanomas was also achieved. Both sensitization to chemotherapeutics and a bystander effect due to CD95L overexpression were shown *in vitro*.

Similarly, melanoma cells turned out as critically sensitive to overexpression of proapoptotic Bcl-2 proteins as shown by transient and stable transfection of Bcl-XS, Bax or Bik/NBK, and the combination with proapoptotic agents as etoposide, pamidronate, and ceramide resulted in additive proapoptotic effects. Also adenoviral expression of Bik/NBK triggered apoptosis in all melanoma cell lines, investigated. In the *in vivo* model, induced expression of Bcl-XS was also able to significantly reduce the size of xenotransplanted melanomas. On the other hand, stable overexpression of Bcl-2 and of Bcl-XL converted apoptosis-sensitive melanoma cells resistant to proapoptotic signals (agonistic CD95 activation, ceramide). With respect to the signalling cascade following overexpression of proapoptotic Bcl-2-related proteins we found strong DNA fragmentation and nuclear condensation after Bcl-XS induction.

Thus, melanoma cells and melanomas in the mouse model can be directly targeted by proapoptotic genes, and death ligands as CD95L or proapoptotic Bcl-2-related proteins represent powerful tools especially against melanoma cells, when transfected or transduced by viral vectors. Selective expression and selective induction of apoptosis can be mediated by applying tyrosinase-derived promoters as we demonstrated by a tyrosinase promoter CD95L construct. Combined use of selective promoters and proapoptotic genes appear as suitable strategies for future melanoma therapies.

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Insights Into Mechanisms Regulating the Growth Response of Melanoma Cell Lines to Vitamin D Analogs *In Vitro*

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Vitamin D analogs inhibit proliferation and induce differentiation in a broad variety of cell types, including human melanocytes. For unknown reasons however, various tumor cell lines fail to respond to the antiproliferative effects of these compounds. We studied the effects of $25(OH)D_3$, $1,25(OH)_2D_3$, and the vitamin D analog seocalcitol (EB 1089) on the growth of various melanoma cell lines (SK-Mel-5, SK-Mel-28, MeWo, MelJuso) using a WST-1 based colorimetric assay. We studied mRNA expression of vitamin D receptor (VDR), vitamin D-25-hydroxylase (25OHase), $25(OH)D_3$-1α-hydroxylase (1αOHase), and $1,25(OH)_2D_3$-24-hydroxylase (24OHase) using real-time PCR. Our results show that the majority of melanoma cell lines analyzed responds to antiproliferative effects of vitamin D analogs (e.g. MeWo), while the minority is nonresponsive (e.g. SkMel5). A strong induction (up to 7000-fold) of 24OHase mRNA was detected in responsive cell lines, indicating functional integrity of VDR-mediated transcriptional activity. In contrast, induction of 24OHase was much lower in nonresponsive cell lines (up to 70-fold). Responsive and nonresponsive cell lines did not differ in VDR-expression, that was induced in both groups up to 3-fold along with vitamin D treatment. However, responsive and nonresponsive cell lines differed in the effects of vitamin D analogs on antiproliferative activity mediated by calpain inhibitors or trichostatin A, an inhibitor of histone deacetylation. Flow cytometry (bcl-2, bcl-xl, bcl-xs, bcl-x, bax, CD95), a cell death detection kit, and analysis of annexin/Pi revealed no induction of apoptosis by $1,25(OH)_2D_3$ (10^{-7} M) or its analogs in responsive or nonresponsive cell lines. We also report the expression of vitamin D-activating enzymes 25OHase and 1αOHase in all cell lines analyzed. Different alternate slice variants of the 1αOHase gene were detected. In conclusion, metastasizing melanoma represents a promising target for palliative treatment with new vitamin D analogs that exert little calcemic side effects and for pharmacological modulation of calcitriol synthesis/metabolism.

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Skin Cancer Prevention, UV-Exposition, and Vitamin D: How Much Sunlight do we Need?

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UV-exposition is the the main reason for the development of skin cancer. In consequence, strict sun protection recommendations represent a fundamental part of skin cancer prevention programs. However, 90% of all requisite Vitamin D is formed within the skin through the action of the sun – a real dilemma, for a connection between Vitamin D deficiency and various types of cancer (e.g. colon-, prostate- and breast cancer) has been confirmed in a whole number of studies. These cancer protective effects of vitamin D most likely depend on extrarenal, local production of $1\alpha,25(OH)_2D_3$, that has been shown in various tissues. Increasing evidence indicates that the lack of sunlight exposure even leads to more than thinning bones and an increased risk for cancer–there are added benefits of vitamin D that include control of blood pressure and cholesterol serum levels. We have now analysed serum 25-hydroxyvitamin D levels in patients under photoprotection, including patients with xeroderma pigmentosum (XP), basal cell nevus syndrome (BCNS), and transplant recipients under immunosuppressive therapy. Serum 25-hydroxyvitamin D levels were decreased in these patients under photoprotection. We conclude that vitamin D serum levels have to be monitored carefully in patients under photoprotection, and that vitamin D deficiency should be treated, e.g. via oral substitution. We discuss our present knowledge about the relevance of vitamin D deficiency for the increased occurrence of certain malignancies and discuss possible consequences for sun protection recommendations that represent a fundamental part of skin cancer prevention programs.

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Modulation of Phosphorylated Histon H2AX (γH2AX) Foci by UV- and X-Irradiation in hMSH2$-/-$ and Wild Type Cells

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Skin tumors are frequently a result of UV-induced DNA damage. The hMSH2 gene plays a central role in DNA mismatch repair. Its promoter has been shown to be upregulated in various cell types in response to UV irradiation due to specific binding of a p53/cJun heterodimer, that we have shown previously. Significance of mismatch repair pathways for the repair of UV-light induced DNA damage was confirmed by demonstrating specific binding of human hMSH2/hMSH6 complexes to DNA incorporating thymine or uracil containing UV-light photoproducts. Furthermore, human cells with mutations in particular mismatch repair genes were likewise found to have a deficiency in transcription coupled repair of UV-induced pyrimidine dimers. Of all the forms of DNA damage, double strand breaks (DSBs) are potentially the most problematic, since they may lead to broken or rearranged chromosomes, cell death or cancer. Increase in phosphorylated histon H2AX (γH2AX) foci have been shown to signal the presence of DNA damage, in particular double strand breaks. hMSH2 deficient tumor cell lines lost most of their ability to accurately repair plasmid DNA double-strand breaks by homologous recombination. UV-light does not directly produce DNA double-strand breaks but rather produces pyrimidin dimers and other photoproducts, that must be removed or bypassed to prevent arrest of the replication fork. UV induced replication arrest in the Xeroderma pigmentosum variant (XPV) but not in normal cells leads to an accumulation of phosphorylated histon H2AX (γH2AX). We have now analyzed effects of UV- and X-irradiation on expression of phosphorylated γH2AX in hMSH2-defective (hMSH2$-/-$) and hMSH2 wild type cells using a specific mAB directed against γH2AX (Trevigen) and immunocytochemical and flow cytometry. We found that X-irradiation induces nuclear foci of γH2AX-immunoreactivity while UV-irradiation results in homogeneous nuclear staining. Our results indicate that hMSH2 status modulates the level of γH2AX in response to irradiation, most pronounced to X-irradiation. While γH2AX immunoreactivity in response to X-irradiation has been shown to correlate with DNA double-strand breaks, our results indicate that γH2AX immunoreactivity in response to UV-treatment correlates with induction of apoptosis.

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E2f1 is a Pivotal Regulator of UVB Induced Apoptosis and Restores Cancer Resistance to Trp53-Deficient Mice

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The Rb-E2f checkpoint is critical for allowing cells to enter S phase; this decision if closely regulated by transcriptional activation of several genes. E2f1 also has a unique pro-apoptotic activity, independent of its control over S-phase entry. Since E2f1 is DNA damage-inducible, we inquired whether its pro-apoptotic function is an element of the known Trp53-dependent pathway for UV-induced apoptosis, important in preventing UV-induced mutations in the epidermis. Contrary to expectation, we find that E2f1$-/-$ mice show enhanced keratinocyte apoptosis after UV. Similarly, epidermis-specific overexpression of E2F1 suppresses UV-induced apoptosis. E2f1$-/-$; Trp53$-/-$ mice exhibit elevated apoptosis similar to that of seen in E2f1$-/-$ alone, a great contrast to the profound apoptosis defect seen in Trp53$-/-$ animals. Double-knockout mice also exhibit a striking annulment of two major developmental aberrations of Trp53$-/-$ mice: the abnormal sex ratio and early-onset tumors. A similar effect of E2f1 is in primary keratinocytes and fibroblasts, but not thymocytes and not after ionizing radiation. These findings demonstrate that E2f1 functions as a suppressor of an apoptosis pathway initiatable by UV and possibly genetic abnormalities. Trp53 opposes this suppression and is dispensable when E2f1 is absent.

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Silencing of Membrane-Type 1 Metalloprotease (MT1-MMP) by Specific siRNAs Suppresses the Angiogenic Phenotype of Dermal Endothelial Cells and Inhibits Type I Collagen Invasion by Melanoma Cells

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Focalized proteolysis by matrix metalloproteinase (MMP) can plays a crucial role in the cell invasive program. The importance of one member of the membrane-anchored MMP subfamily, i.e. MT1-MMP (MMP-14) in angiogenesis and melanoma invasion was recently delineated. Indeed, MT1-MMP is involved in pericellular collagenolysis, pro-gelatinase A (MMP-2) and $\alpha v\beta 3$ integrin activation and CD44 or LRP shedding events and therefore might be considered as one main target in skin cancer. We thus developed the siRNA approach to invalidate MT1-MMP expression in dermal endothelial cells and invasive melanoma cells (M3Da). Among siRNAs tested, only siRNAs (25–50 mM) targeted regions 107–207 and 228–248 of MT1-MMP mRNA synthesized by *in vitro* transcription proved to inhibit by >80 per cent MT1-MMP expression at the mRNA and protein levels in both cell types. It also impaired PMA-mediated pro-Gelatinase A (MMP2) activation in endothelial cells. Elastin fragments were recently reported to stimulate the rate of pseudotubes formation by endothelial cells in the Matrigel® assay and to increase the invasion of type I collagen by melanoma cells in a Transwell® model, both through MT1-MMP up regulation. Preliminary data indicated that MT1-MMP $mRNA_{107\text{-}207}$ transfection of endothelial cells and M3Da cells abolished the elastin-induced angiogenic phenotype and collagen invasive capacity, respectively.

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Transfection with siRNA Suppresses Expression of DNA-Mismatch Repair Enzyme *h*MSH2 in Melanoma Cell Lines Thereby Modulating Repair of UV-Induced DNA-Damage as well as UV- and Chemotherapy-Induced Apoptosis and Cell Death

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Human MSH2 is a well characterized component of the DNA repair system. The MSH2 gene is part of the post-replicative mismatch repair system that prevents the accumulation of spontaneous mutations and, thereby, ensures the integrity and stability of the genome. It is the most frequently impaired gene in hereditary nonpolyposis colorectal cancer (HNPCC). Ultraviolet (UV) radiation plays a pivotal role in skin damage and photocarcinogenesis. The mechanism how *h*MSH2 is involved in the complex response mechanisms to UV damage is not completely clear. We have now analyzed the importance of *h*MSH2 for UV- and chemotherapy-induced apoptosis and cell death. Using quantitative "real time" PCR, we detected strong *h*MSH2 mRNA expression in melanoma (MM) cell lines. In contrast to a cutaneous squamous carcinoma cell line (SCL-1) that showed considerable activation of the promoter and increase of *h*MSH2 mRNA in cells after UVB treatment, MM cell lines revealed only weak differences in *h*MSH2 mRNA and protein amount in response to UV-B. Transfection of *h*MSH2 siRNA into human melanoma cells resulted in a dose-dependent inhibition of *h*MSH2 expression (up to 80%). When pCMV-β-Gal-plasmid DNA was irradiated with 1 kJ/m^2 UV-B and transfected to melanoma cells, β-Gal activity was reduced in *h*MSH2 siRNA treated cells as compared to untreated cells. When UV-B-irradiated pCMV-β-Gal-plasmid DNA was transfected in MEF *h*MSH2 wt vs. $-/-$ cells, ß-Gal activity was 20% reduced in $-/-$ cells as compared to wt cells after 24 h. Transfection of *h*MSH2 siRNA into human melanoma cell lines resulted in an increase of UV-B-induced apoptosis and in increased chemosensitivity. Our results suggest that *h*MSH2 may be of importance for pathogenesis of MM and that *h*MSH2-targeted siRNAs are effective inhibitors of *h*MSH2 expression and may have therapeutic potential in the treatment of metastasizing MM by themselves or as chemosensitizers in combination with other compounds.

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RhoC as a Determinant of Metastases in Human: Potential Downstream Targets

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Over-expression of RhoC in various human cancers has been correlated with the metastatic stage of tumor progression and has also been identified as one of the genes that enhance metastasis in both human and mouse melanoma lines (Clark *et al*, 2000, Nature). The purpose of this work was to understand how RhoC over-expression contributes to human melanoma metastasis.

We have established stable transfectants over-expressing RhoC, using human melanoma cell lines representing distinct stages in melanoma progression: WM35, WM278 and WM1617, representing radial growth phase, vertical growth phase and metastatic phase in melanoma progression respectively. To identify potential downstream targets of over-expression of RhoC in WM35 cells in a global way, we performed Affymetrix microarray assays using human U133A chip. We identified 73 potential genes as downstream targets of RhoC over-expression. Some of these genes are tumor progression markers, and some of them have functions in migration, detachment, and invasion of cells. We have used functional invasion and attachment assays to verify the importance of these genes.

Unexpectedly, we also found that some of the genes are involved in immune surveillance. Our preliminary FACS analysis confirmed that over-expression of RhoC down-regulated these genes involved in immune surveillance. These data suggest that RhoC over-expression might promote melanoma metastasis in part by enhancing tumor immune escape. These studies identified a potential novel way for over-expressing RhoC to promote melanoma metastasis.

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BRAF Kinase Gene V599E Mutation in Growing Melanocytic Lesions

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Mutations in the BRAF-gene are found in benign and malignant melanocytic lesions, >90% being a V599E mutation. This mutation results in constitutively active kinase function and increased colony formation *in vitro*. The biological impact of this mutation *in vivo* is still debated. To address this question, we used our digital epiluminescence image archive and retrospectively selected 49 melanocytic lesions, which did not meet the criteria of melanoma at the initial presentation. Mean 12 months later these lesions were excised because of increased size or changed structure and BRAF^{V599E} mutations were analyzed. Among 36 growing lesions, BRAF^{V599E} mutations were found in 16 (11 melanomas and 5 nevi). Among 13 lesions with structural changes, BRAF^{V599E} mutations were found in 4 (3 melanomas and 1 nevus). 35 randomly selected additional lesions with no changes during follow-up served as controls, all nevi by histology, and 2 of them showed a BRAF^{V599E} mutation. Statistics revealed odds for the presence of the BRAF^{V599E} mutation being 7 times higher in lesions with structural changes and 13 times higher in growing lesions as compared with lesions without changes. This raises the question if the V599E mutation determines lesions at risk developing into melanoma and if not, what is the mechanisms controlling growth stop in benign lesions.

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Nerve Growth Factor Receptors Modulates Apoptosis in Melanoma Cell Lines

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Melanocytes are neural crest-derived cells and respond to nerve growth factor (NGF) stimulation by increased dendricity. It has been shown that neurotrophins (NT) are potent inducer of melanoma brain metastases, in that they exert chemotactic activity for human melanoma cells. However, little is known on the signaling mechanism underlying this activity, and in particular the role of both the low-affinity (p75NTR) and the high-affinity (trk) NT receptors remains to be clarified. As the balance between p75NTR and trk determines survival or death in neuronal and non-neuronal cells, we wanted to evaluate the expression and function of NT receptors in malignant melanoma. To this purpose, several melanoma cell lines from both primary and metastatic melanomas were cultured with or without the addition of the chemotherapeutic agent carmustine. We first demonstrated that A375, Mewo, Me 272, WM115, WM266-4 and SK-MEL-4 cell lines express p75 and the high-affinity NGF receptor trkA mRNA and protein, though at different levels, by PCR and western blotting. By contrast, melanoma cell lines do not express mRNA of trkB and trkC, the high-affinity receptor of brain-derived neurotrophic factor (BDNF) and NT-3, respectively. We also confirmed by flow cytometry that melanoma cell lines express different levels of p75, and in particular 21% in A375, 48% in Mewo, 0.7% in 272, 64% in WM115, 75% in WM266-4 and 12% in SK-mel4. WM115, WM266-4 and SK-mel4 cell lines underwent apoptosis upon treatment with carmustine (50 μg/ml) at 48 hrs ($p<0.01$). The degree of cell death (WM115 = 55%; WM266-4 = 69%; SK-mel4 = 47%) well correlated with the level of the death receptor p75NTR. In addition, carmustine up-regulated the expression of p75 by western blotting in many cell lines, including WM115 and WM266-4, but not in SK-mel-4. On the other hand, carmustine reduced trkA levels in SK-mel-4 as well as in other melanoma cell lines. These results seem to suggest that the balance between p75NTR and trkA influences the apoptotic signal induced by the chemotherapeutic agent carmustine in melanoma.

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UVB Fingerprint Mutations at the *INK4a-ARF* Locus in Merkel Cell Carcinoma

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Merkel cell carcinoma is rare tumor of putative neuroendocrine origin, primarily affecting elderly persons at sun-exposed sites. The mortality rate of this tumor is substantial and the 5-year survival rate is about 50 to 70 percent. Similar to non melanoma skin cancers, especially squamous cell carcinoma, exposure to ultraviolet (UV) radiation seems to be a risk factor for Merkel cell carcinoma. However, the exact molecular mechanisms by which UV radiation may lead to the formation of Merkel cell carcinoma remain to be determined. For instance, unlike in squamous cell carcinoma typical UV(B) fingerprint mutations (i.e., C to T transitions at dipyrimidine sites) at the tumor suppressor gene *p53* are not frequent in Merkel cell carcinoma. Thus, other genes than *p53* may be the primary targets for Merkel cell carcinogenesis. One candidate gene is *INK4a-ARF*. This locus on the human chromosome 9p21 encodes the alternative reading frame proteins p16INK4a (exons 1-alpha, 2, and 3) and p14ARF (exons 1-beta, 2, and 3), both of which are involved in the negative control of cell proliferation by a tumor suppressor function. In this study, we analyzed Merkel cell carcinomas from 15 patients (13 females and 2 males; mean age, 79 years; age range, 77 to 88 years). Direct sequence analysis of the *INK4a-ARF* locus revealed a total of 9 missense mutations in 7 of 15 (47%) tumors. Six of 9 (67%) mutations were C to T transitions at dipyrimidine sites (i.e., UV fingerprint mutations). Six of 9 (67%) mutations affected exon 1-alpha and 3 (33%) mutations exon 2. All 9 mutations affected the amino acid sequence of p16INK4a and one affected both p16INK4a and p14ARF. Taken together, our results suggest that UV(B) causes mutations at the INK4a-ARF locus, mainly affecting the p16INK4a protein. These mutations may play a causative role in the tumorigenesis of a substantial portion of Merkel cell carcinomas.

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Specific Immune Responses to HPV5 Antigens in Patients with Epidermodysplasia Verruciformis (EV) Associated with Mutations in *EVER2* Gene

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Recently, nonsense mutations in two novel genes (*EVER1* and *EVER2)* have been identified in patients with EV, i.e. cutaneous disease characterized by an abnormal susceptibility to human papillomaviruses (HPVs). These genes belong to a transmembrane channel-like (*TMC*) gene family, and transmembrane EVER proteins seem to be tightly coupled to the same pathway in the endoplasmic reticulum suggestive of their involvement in the immune control of HPV infection. Therefore the aim of the study was to find out cell-mediated immunity and cytokine production in response to HPV5 VLPs (virus-like particles) in patients with EV and *EVER2* mutations (n = 5) as compared to healthy controls (n = 10). T lymphocytes were cultured for 14 days in the presence of HPV5a VLPs and IL-2, and studied for their proliferative responses to the VLPs presented by freshly isolated, X-ray irradiated peripheral blood mononuclear cells (PBMC). We found that T lymphocytes from EV patients with *EVER2* mutations, in contrast to the healthy subjects, did not respond to HPV5a VLPs, as measured by the proliferative assay and IFNγ production. PBMC from EV patients, when stimulated with HPV5 VLPs produced significantly higher level of IL10 and decreased amount of IFNγ, as compared to controls. To find out whether this defect could be reversed, we studied local and systemic cell mediated immunity and production of specific antibodies after intradermal application of highly purified HPV5 VLPs. In one patient local CMI became positive and in this case the initially lowered IFNγ production by PBMC was found increased after VLP administration. Stimulation of specific immunity with HPV5 VLPs was found also beneficial for prevention of EV progression in early familial cases, as shown in a granddaughter of an EV patient with a severe form of the disease. The study showed that in spite of *EVER2* mutations, found in all our patients, the specific immune defect could be partially overcome by HPV5 VLP stimulation.

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Stable Overexpression of Smad7 in Human Melanoma Cells Specifically Alters their Invasive Phenotype *In Vitro*

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Previous identification of constitutive Smad signaling cascade activation in human melanoma cells despite resistance to TGF-beta control of cell proliferation lead us to investigate the effect of inhibitory Smad7 overexpression on melanoma cell behavior. Stable expression of Smad7 resulted in reduced Smad3/4 binding to DNA when cells were treated with TGF-beta, accompanied with drastically reduced Smad3/4-driven gene transactivation, as measured using transfected Smad3/4-specific reporter gene constructs. Smad7 overexpression, however, did not alter the proliferative capacity and resistance to TGF-beta-driven growth inhibition, nor the resistance to drug-induced apoptosis, of several human melanoma cell lines. On the other hand, dramatic reduction in their capacity to invade Matrigel or to cross a collagen gel placed onto a porous membrane in a Boyden migration chamber was observed. Gelatin zymography identified reduced MMP2 and MMP9 production by Smad7-transfected melanoma cells as compared to mock-transfected cells. Work is in progress to determine whether Smad7 overexpression alters melanoma cell capacity to (a), form tumors in nude mice when injected subcutaneously, and (b), to metastasize to the lungs.

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Association of Novel and Stable Translocations with Oncogene Expression in Malignant Melanoma

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Cytogenetics has not only precipitated the discovery of several oncogenes, but also led to the molecular classification of numerous malignancies. The correct identification of aberrations in many tumors has, however, been hindered by extensive tumor complexity and the limitations of molecular cytogenetic techniques. In this study we have investigated 5 malignant melanoma cell lines from at least three different passages using high resolution R-banding and the recently developed methods of CGH, and M-FISH. We subsequently detected nine consistent translocations, seven of which were novel: dic(1;11)(p10;q14), der(9)t(3;9)(p12;p11), der(4)t(9;4;7)(q33::p15-q23::q21), der(14)t(5;14)(q12;q32), der(9)t(9;22)(p21;q11), der(19)t(19;20)(p13.3;p11), der(10)t(2;12;7;10)(q31::p12 → pter::q11.2 → q31::q21), der(19)t(10;19)(q23;q13) and der(20)t(Y;20)(q11.23;q13.3). Furthermore, using the human HG-U133A GeneChip, positive expression levels of oncogenes or tumor related genes located at the regions of chromosomal breakpoints were identified including AKT1, BMI1, CDK6, CTNNB1, E2F1, GPNMB, GPRK7, KBRAS2, LDB2, LIMK1, MAPK1, MEL, MP1, MUC18, NRCAM, PBX3, RAB22A, RAB38, SNK and STK4 indicating an association between chromosomal breakpoints and altered gene expression. Since the majority of these breakpoints have been reported previously in malignant melanoma, our results support the idea of common mechanisms in this disease.

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Distinct Types of Primary Cutaneous Large B-Cell Lymphoma Identified by Gene Expression Profiling

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In the EORTC classification two types of primary cutaneous large B-cell lymphoma (PCLBCL) are distinguished: primary cutaneous follicle center cell lymphomas (PCFCCL) and PCLBCL of the leg (PCLBCL-leg). Distinction between both groups is considered important because of differences in prognosis (5-year survival $>95\%$ and 52%, respectively) and the first choice of treatment (radiotherapy or systemic chemotherapy, respectively), but is not generally accepted.

To establish a molecular basis for this subdivision in the EORTC classification we investigated the gene expression profiles of 21 PCLBCL by oligonucleotide microarray analysis. Hierarchical clustering based on a B-cell signature (7450 genes) classified PCLBCL into two distinct subgroups consisting of respectively 8 PCFCCL and 13 PCLBCL-leg. PCLBCL-leg showed increased expression of genes associated with cell proliferation, the proto-oncogenes Pim-1, Pim-2 and c-Myc, and the transcription factors Mum1/IRF4 and Oct-2. In the group of PCFCCL high expression of SPINK2 was observed. Further analysis suggested that PCFCCL and PCLBCL-leg have expression profiles similar to that of germinal center B-cell-like and activated B-cell-like diffuse large B-cell lymphoma, respectively. The results of this study suggest that different pathogenetic mechanisms are involved in the development of PCFCCL and PCLBCL-leg and provide molecular support for the subdivision used in the EORTC classification.

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Expression Pattern of Fibroblast Activation Protein (FAP) and Endosialin/TEM-1 (Tumor Endothelial Marker 1 in Stromal Compartments of Human Skin Cancers

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Growth and metastasis of solid neoplasms require the recruitment of a supporting tumor stroma. A highly consistent trait of tumor stromal fibroblasts in most epithelial cancers and, as recently demonstrated, in melanocytic skin tumors, is the induction of fibroblast activation protein (FAP), a member of the serine protease family. Endothelial cells represent another important cell type of the reactive tumor stroma. The cell surface glycoprotein endosialin is selectively expressed by tumor blood vessel endothelium in a broad range of human cancers. Endosialin is identical to TEM-1 (Tumor Endothelial Marker 1), which has been linked to tumor endothelium by serial analysis of gene expression (SAGE) profiling.

The present study was designed to (i) analyze the protein expression pattern of FAP and endosialin in a series of melanocytic and epithelial skin tumors and (ii) to test the suitability of *in silico* derived cancer associated profiles for the identification of tumor stroma relevant genes, by comparing the protein expression patterns of FAP and endosialin with transcript profiles derived from in silico analysis.

FAP-positive fibroblasts were detected immunohistochemically in variable amounts in the reactive stromal fibroblasts of all the tissues tested, including cases of melanocytic nevi ($n=28$), metastatic melanoma ($n=22$), basal cell carcinoma ($n=7$) and squamous cell carcinoma ($n=2$). Two distinct FAP-expressing stroma compartments were identified in melanoma metastases: a strong peritumoral stroma compartment and a patterned intratumoral compartment in the immediate vicinity to the tumor cells. FAP induction was also seen in dermal fibroblasts in morphologically normal skin adjacent to melanoma metastases. Expression of Endosialin was restricted to subsets of small and medium sized tumor blood vessels in metastatic melanomas and in the two SQCC tested. In addition, scattered endosialin-positive stromal fibroblasts were seen in many of the cases including melanocytic and non-melanocytic tumors. Analyzing gene expression data from proprietary databases, we found a direct correlation between protein expression and mRNA levels for FAP in human skin cancers. For endosialin, changes in expression levels could not be detected by in silico analysis, as the specific signals derived from tumor endothelial cells are masked due to the heterogeneity of the tissue samples (tumor cells + stroma + blood vessels).

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K5-SOS-F Induce Increased Migration in Mouse Primary Keratinocytes

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K5-SOS-F transgenic mice develop skin tumors dependent on the presence of a functional epidermal growth factor receptor (EGFR), which provides an essential survival signal in these tumors. We have characterized primary keratinocytes from K5-SOS-F transgenic mice for their adhesive properties and the expression of downstream signaling molecules. We found that the protein levels of the non-receptor protein tyrosine kinase Src and of the adhesion molecule β1-integrin are increased in primary keratinocytes expressing K5-SOS-F. Since Src is a downstream signaling protein of many membrane receptors including EGFR and integrins, it could play an important role in the crosstalk between both these receptors. First experiments show that the adhesion of keratinocytes to collagen might be EGFR-dependent since in an EGFR hypomorphic (wa2) background the adhesion to collagens is reduced but can be rescued by expression of the K5-SOS-F transgene. An increased polarization of actin filaments and a decrease in the number of focal adhesions is also observed in K5-SOS-F transgenic keratinocytes. Consistent with these observations, we also found an increased migration of K5-SOS-F transgenic keratinocytes which is abolished by inhibition of EGFR, MAP kinase and Src with specific inhibitors. Moreover, the increased migration of these keratinocytes is growth factor-dependent since under serum free conditions migration is inhibited and can only partially be rescued by the addition of EGF. Therefore, it seems that EGFR-dependent tumor formation in K5-SOS-F transgenic mice is not only caused by increased survival but also by increased migration of transgenic keratinocytes. We are currently investigating how the increased protein levels of Src and β1-integrin are affecting adhesion and migration of K5-SOS-F transgenic keratinocytes and the results of these experiments will be presented.

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Expression and Phosphorylation of Src-Type Tyrosine Kinases in Cutaneous T Cell Lymphoma (CTCL)

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Mycosis fungoides (MF) and its leukemic variant Sézary syndrome (SS) are the most frequent types of cutaneous T cell lymphomas (CTCL). We recently found that CTCL cells contain constitutive NFkB activities, which could be down-regulated by agents that inhibit src-type tyrosine kinases. The src gene family contains members that mediate T-cell receptor signals and known oncogenes.

We therefore investigated MF and SS cell lines and skin lesions for src-type tyrosine kinase expression by Western blotting and Immunohistochemistry respectively. Western blot experiments with antibodies specific for c-src, c-fgr, c-yes, lck, and fyn showed that c-src, c-yes, and fyn were expressed in all four tested cell lines. The T-cell receptor associated src-type kinase lck was expressed only in one cell line, indicating that this protein was not involved in NFkB activation in MF and SS cell lines. The c-fgr gene was expressed in no cell line. When we tested MF and SS skin lesions for the expression of the c-src and c-yes oncogenes, we found that c-src was already expressed in early (patch/plaque) stages, whereas c-yes was only expressed in late (tumor) stages. Since c-Src has to be activated by phosphorylation, we also tested CTCL skin lesions for phosphorylated c-Src and found that activated c-Src is present only in the tumor stage.

Our results show that the c-src and c-yes tyrosine kinases, which are not expressed in normal T cells, are present in MF and SS cells. Since src-type tyrosine kinase inhibitors like herbimycin A and PP2 inhibit the constitutive NFkB activities, we suppose that c-src and c-yes are involved in constitutive activation of NFkB in MF and SS cells, which in turn may lead to the expression of further genes that promote the cancerogenesis of CTCL.

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DNp63 Transcription is Regulated by p53 and p73 upon DNA Damage in Keratinocytes

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The p63 gene belongs to the p53 gene family of tumor suppressor genes and encodes for sequence specific transcription factors that act either as activators or as repressors of transcription. The expression of a specific isoform of the p63 gene, namely the DNp63a isoform, is thought to identify the cells belonging to the staminal compartment of the epidermis and cutaneous adnexa and was found to be upregulated in squamous cell carcinomas. Indeed DNp63a may act as a master controller of staminal keratinocytes proliferation by regulating the transcription of specific genes involved in cell cycle arrest and differentiation such as the cdk inhibitor p21 and the 14-3-3sigma genes. DNp63a is also regulated by DNA damage: UV irradiation induces its phosphorylation and causes the detachment from cell cycle related and apoptosis related p53 dependent promoters. We have investigated the effects of other kind of DNA damaging agents in the regulation of DNp63 in keratinocytes and found that DNp63 transcription is tightly regulated by other members of the p53 family being repressed by the activatory TA isoforms and activated by DN isoforms. DNp63 expression is also regulated by the mutational status and by the polymorphism at position 72 of the tumour suppressor gene p53. The selective interference with specific components of this activatory/repressory loop leads to significant changes in DNp63 expression and in the biological outcome of DNA damage. Taken together our data suggest that modulation of DNp63 transcription by the other members of the p53 family may contribute to cell transformation and to the resistance to chemotherapy in skin cancer.

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Identification of Novel Non-Melanoma Skin Cancer Molecular Markers by Micro Array Technology

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Objective. Carcinogenesis is a multi-step process indicated by several genes up- or down-regulated during tumor progression. The type and number of genes involved in human non-melanoma skin cancer (NMSC) are still unclear. This study examined and identified different expressed genes and pathways involved in this process.

Patients and Methods. Fifteen snap-frozen biopsies of 5 immunosuppressed organ-transplanted recipients each normal skin, actinic keratosis (AK) and invasive squamous cell carcinoma (SCC) were collected and analysed. All 5 extracted RNA specimens of normal skin and 2 of AK were pooled. In addition, we have included 5 normal skin specimens from age-matched non-immunosuppressed patients. Thus, we have examined 6 normal skin, 4 skin pre-cancer (AK), and 5 skin cancer (SCC) and all biopsies were confirmed by histology. Total RNA was used for hybridization with Affymetrix HG-U133A gene expression DNA-microarrays containing 22,283 known genes. Confocal scanner evaluated the signals twice and data analysis was performed by bioinformatics. The cut-off was more than 2.0-fold changes in gene expression confirmed by *t* test. Quantitative verification of 13 up- or down-regulated genes was performed by real-time RT-PCR and genes were confirmed by sequencing.

Results. Broad coherent patterns in normal skin *vs* AK and/or SCC were observed for 119 genes. Some of the genes (e.g. annexin, metalloproteinases, and lamin) up-regulated in carcinomas in our study have previously reported as over-expressed in NMSC. In total 44 genes were up-regulated and 75 genes were down-regulated in non-melanoma skin cancer, including proliferation and cell cycle antigens, transcription factors, growth factors, and genes regulating the immune system. Eighty-five percent (11 of 13) of verified genes by real-time RT-PCR showed significant different expression ($p < 0.05$) in normal skin tissue *vs* skin (pre)cancer.

Conclusions. The majority of genes identified have not been previously reported different expressed during non-melanoma skin carcinogenesis. Microarray analysis of human skin biopsies without microdissection allows the identification of differentially expressed genes that may prove useful as early detection or progression markers.

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Fluorescence *In Situ* Hybridisation (FISH) Analysis of Chromosome 7 and 8 Aberrations in Cutaneous T-Cell Lymphomas

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In cutaneous T-cell lymphomas (CTLC), a characteristic pattern of chromosomal aberrations (6q-7-8q-13q) was described. In the present study, chromosomes 7 and 8 were screened for numerical and structural aberrations in 19 patients with CTLC using fluorescence *in situ* hybridisation (FISH). Centromeric probes were used for chromosome 7 and 8. For the analysis of candidate tumor genes probes for the regions 7q34-35 (encoding the T-cell receptor beta region) and 8q24 (encoding the myc-oncogen) were chosen. Minimum and maximum signals of 2–4% of labeled nuclei were regarded as a cut of levels for the probes, respectively. For chromosome 7 centromeric probes, aberrations were observed in 8 of 19 patients, for chromosome 8 in 9 out of 19 patients. The patients identified by CGH were confirmed by FISH and, moreover, additional patients were identified. 6 out of 10 patients revealed aberrations of the region 7q34-35, and 4 out 10 patients for 8q24. Sequential analysis of tumor samples obtained from patients during the course of the disease revealed a consistent occurence of the aberrations. The results of this study demonstrate that FISH analysis of tumor samples of CTLC is a reliable and reproducable technique. The prognostic relevance of frequent aberrations remains to be determined.

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Thalidomide Combined with Dacarbazine Enhances the Anti-Tumor Activity in a Human Melanoma Xenotransplantation Model

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Malignant melanoma is a well known example of a tumor responding poorly to conventional adjuvant treatment modalities including chemotherapy. Anti-angiogenic therapy represents a promising treatment modality for various human cancer. For thalidomide it has been demonstrated that its anti-angiogenic properties result in clear anti-tumor activity in multiple human malignancies. Furthermore, combinations of anti-angiogenic drugs and chemotherapeutics are an increased applied treatment strategy for multiple malignancies. In human melanoma, little is known about the therapeutic potential and mechanism of thalidomide. Recently, a Phase II showed promising results combining thalidomide and temozolomide for the treatment of patients with human melanoma.

To determine the anti-tumor activity and the mechanism of action we studied thalidomide in a human melanoma SCID mouse xenotransplantation model. Single agent activity of thalidomide as well as combination with dacarbazine (DTIC), still the single most effective agent used in melanoma therapy, were tested.

Thalidomide combined with dacarbazine treatment markedly enhanced the anti-tumor effect and showed a significant tumor reduction relative to the dacarbazine-only group (61%) and even more tumor reduction (74%) compared to the solvent group. A significantly lower microvessel density was encountered in the thalidomide treatment groups (thalidomide alone or combined with DTIC) underscoring the anti-angiogenic effect of thalidomide in this model. In line with these results, we observed a nearby three fold increase of apoptosis for the combination of thalidomide and DTIC. These data underline the rationale for combining dacarbazine – a cytotoxic agent – and thalidomide – an anti-angiogenic cytostatic agent as a promising option for treatment of malignant melanoma.

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5-Fluoruracil Treatment Alters Ferredoxin-reductase-mRNA Splice Variant Ratios in Colorectal Cancer Patients: Impact on Therapeutical Response

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Success rates of 5-Fluoruracil (5-FU) based chemotherapy remain still low. Recently, it has been reported that both mRNA and protein levels of ferredoxin reductase (FDXR) increased drastically after 5–FU treatment in colorectal cancer cell lines (HCT 116). Therefore, we hypothesized that induction of the ferredoxin reductase mRNA levels may be a good marker for successful 5-FU chemotherapy. Fourty patients with advanced colorectal carcinoma were treated with 5′DFUR – a prodrug of 5-FU – for 14 days. Biopsies of tumor and surrounding normal tissues were collected before treatment, and surgical specimens of tumor and normal tissues were taken at the time of tumor removal. FDXR-mRNA levels of the two alternative splice variants characterized by the presence ($+18$-form) or absence of 18 base pairs (–18-form) were estimated by real-time PCR. These two forms are usually found at a ratio of 100 to 1, irrespective of the tissue studied. The therapeutical success was evaluated by histological investigations of the tissues before and after treatment ($n = 40$). Prior to treatment, total FDXR mRNA levels and the –18–form were about 2-fold higher in normal tissues compared to the tumor for ($n = 38$), while the $+18$-form was slightly increased in tumors (1.9). The previously reported 100:1 ratio for the splice variants or even higher ratios were found in 44.4% of normal tissues, and 94.7% of the tumors studied. 5-FU-prodrug treatment for 14 days did not change levels of the -18-form significantly. In the 40 cases studied by us, we did not detect the above mentioned large increase of FDXR-mRNA levels from *in vitro* studies (Hwang, Bunz *et al*, 2001). Unexpectedly, ratios of $+18$-and –18-form were shifted by 5-FU treatment towards the $+18$-form both in tumors (18-fold increase), and in normal tissues (31-fold). In a subgroup ($n = 5$), the levels of the $+18$-form were similar to -18-form (ratio $-18/+18$ between 0.1 and 2). The latter was clearly associated with resistance to 5-FU treatment.

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Elastin-Derived Peptides Upregulate IL-1β Expression in Melanoma Cells

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In a previous work, we reported an up-regulation of matrix metalloproteinases -1 and -2 (MMP-1 and -2) expression and activation in melanoma cells *in vitro* and showed the contribution of elastin-derived peptides (EDPs) in these effects. We hypothesized that EDPs, following interactions with elastin receptor, could modulate expression of other mediators involved in cancer progression. Thus, in this study, we investigated the contribution of EDPs in cytokines expression.

Our results first evidenced that EDPs treatment of melanoma cells presenting a high tumorigenic potential (M_3Da cells) led to a strong and rapid enhancement in IL-1β mRNA expression whereas GRO-1 mRNA was barely affected. The effects of EDPs on those melanoma cells were found to be mediated, at least in part, by receptor occupancy, as being suppressed by lactose and reproduced by cell stimulation with the VGVAPG peptide. The role of transduction pathways in mediating this effect was assessed with the specific chemical inhibitors U0126, SB203580, LY294002 of the Erk1/2, p38 and PI3K pathways respectively, using western-blot analysis. Binding of EDPs to their receptor induced a prolonged activation of Erk and a transient activation of p38 but not PI3K pathways. Concomitantly, we demonstrated that stimulation by EDPs affected the regulation of AP-1, NF-κB and SP-1 transcription factors. Upon EDPs stimulation, nuclear translocation and DNA binding of AP-1 and NF-κB were enhanced. Interestingly, SP-1 nuclear translocation was reduced.

Comparison of potential responsive elements from the different cytokine promoters underlined the prominent role of transcription factors in selective cytokine expression. On this basis, it is possible that EDPs activation of NF-κB is responsible for IL-1β up-regulation on tumorigenic melanoma cells whereas, a combined-antagonized effect of NF-κB and SP-1, which is down-regulated, would explain the lack of responsiveness of GRO-1 promoter. Further examinations are still needed to unravel the molecular mechanisms induced by EDPs and evaluate the precise role of each transcription factor in the expression of a specific cytokine.

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Differential Expression of the Lymphatic Marker Podoplanin in Human Squamous Cell Carcinomas and Basal Cell Carcinomas

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The mucin-type glycoprotein podoplanin is specifically expressed by lymphatic, but not blood vascular endothelial cells, in culture and in tumor-associated lymphangiogenesis. Our previous studies showed that podoplanin deficiency results in congenital lymphedema and impaired lymphatic vascular patterning, and that podoplanin enhances migration and adhesion of endothelial cells by reorganization of the cytoskeleton. However, its expression in normal tissues and in human malignancies has remained unknown. Using tissue arrays of normal human tissues and of a number of human cancers, we found that – in addition to lymphatic endothelium – podoplanin is also expressed by bile duct cells of the liver, peritoneal mesothelial cells, osteocytes, glandular myoepithelial cells, ependyma cells, and by stromal reticular cells and follicular dendritic cells of lymphoid organs. In normal human skin podoplanin is focally expressed by basal keratinocytes, as shown using different antibodies. Immunohistochemical staining of paraffin embedded tissues revealed strongly induced podoplanin expression in 62 out of 77 squamous cell carcinomas (SCC). The expression pattern was dependent on the level of differentiation of the tumors. In contrast, no podoplanin expression was observed in 20 basal cell carcinomas. Activation of the epidermal growth factor receptor (EGFR) has been proposed to enhance the metastatic potential of SCC, and podoplanin expression is known to be increased by EGF in keratinocytes *in vitro*. Therefore, based on the present findings of podoplanin expression in SCC – but not in BCC – we conclude that podoplanin may play an active role in epithelial tumor progression.

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Differential Effects of Class I/II Versus Class III Histone Deacetylases Inhibitors (HDACi) in Primary Sezary' Cells

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Remodeling of chromatin as well as the dynamic changes at the nucleosome level play a key role in the regulation of gene expression influencing cellular proliferation, differentiation and apoptosis. One of the most important mechanisms implicated in chromatin remodeling is the post-trascriptional modification of the histones by acetylation of their N-terminal tails. The acetylation is mediated by enzymes with acetyl-transferase activity (HAT) while deacetylation is mediated by histone deacetylase enzymes either of the HDAC family or of the Sir2 family. The alteration of both histone acetylation and deacetylation mechanisms are often involved in the pathogenesis of tumors. The histone deacetylase inhibitors represent a new class of antitumoral drugs capable of modulating the ratio between acetilation/deacetylation intensifying in that way the activity or a great number of antineoplastic drugs driving, previously resistant cells, to apoptosis. Valproic acid (VPA), is a drug largely used in the long term treatment of epilepsy, und demonstrates a strong inhibitory activity towards the deacetylases of the HDAC class while Nicotinamide and Sirtinol inhibit the Sir2 deacetylases family. We have studied the anti-neoplastic activity of deacetilase inhibitors in the Sezary' Sindrome cell line (HUT-78) and in Sezary' cells isolated from a patient in the early stage of the disease. The treatment with Valproic acid has induced high levels of apoptosis in both cell lines studied. Moreover low doses of VPA sensitized, these previously resistant cells, to apoptosis induced by cisplatin. On the other side the deacetylase inhibitors of the Sir2 family have induced the cell-cycle arrest without influencing the levels of apoptosis. Our results suggest that the deacetylase inhibitors of the HDAC class may be useful in order to enhance the effect of the chemotherapic drugs commonly used in the treatment of cutaneous lymphomas.

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New Frequent Polymorphism 1765 C->T in the Gene Coding Transcriptional Repressor Oncoprotein Gfi-1 in Cutaneous T-Cell Lymphomas (CTCL)

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Introduction: Gfi-1 encodes transcriptional repressor oncoprotein identified in a retroviral insertion mutagenesis screen for tumor progression in rat T-cell lymphoma lines. The aim of our study was to test the association of new identified 1765 C->T polymorphism with CTCL in the Czech population.

Materials and methods. The heteroduplex analysis followed by capillary as well as radioisotopic sequencing identified alteration in Gfi-1 non-coding 3′ sequence that proved to be the 1765 C->T transition. As a following step, we developed a simple restriction analysis method using AvrII enzyme that enables the rapid detection of this new polymorphism. The genotype distributions as well as allelic frequencies of the polymorphism were compared between 61 patients with CTCL and a group of 93 healthy individuals of similar age and gender distribution.

Results. No significant difference in allelic frequencies of examined polymorphism 1765 C->T in patients with CTCL (T = 36.9%) against the controle subjects (T = 44.1%; p = 0.209) was proved. We did not found any significant difference in the genotype distributions of the examined polymorphism between the patients with CTCL and the controls (p = 0.227). However, the odds ratio of the TT genotype in CTCL patients was 0.546 (95% confidential interval = 0.22-1.14, p = 0.07). Therefore, the TT genotype of 1765 C->T polymorphism of Gfi-1 gene may have potentially protective effect against CTCL.

Discussion. Gfi-1 is supposed to be able to act as a dominant oncogene and cooperates in the process of lymphomagenesis with Myc and Pim-1. It is likely to inhibit apoptosis by means of its repression of multiple proapoptotic regulators. The allelic variant T od 1765 C->T polymorphism is characterized by extinction of the binding site for AP-1 protein in 3'region of Gfi-1 gene. From this point of view, it is interesting that the homozygote genotype of this allelic variant (TT) seems to be associated with lower OR for CTCL.

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Cutaneous T Cell Lymphoma and Toll-like Receptors

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Cutaneous T cell Lymphoma (CTCL) is characterized by a proliferation of activated CD4+ T cells in the skin. The origin of this pathology remains unknown but the implication of viruses is highly suspected. Toll-like Receptors (TLRs) are innate immune receptors. They constitute the first line against infections and they trigger the specific immune response. TLR 2, TLR 4 and TLR 9 are expressed by keratinocytes.

The aim of this work was to look for a new way of T cells activation in CTCL, in particular the keratinocytes TLRs expression in this pathology. In a first part, the expression of TLR 2 (GRAM+specific), TLR 4 (LPS and GRAM–specific) and TLR 9 (viral and bacterial DNA specific) was studied by immunohistochemistry on cutaneous section of CTCL patients before treatment. This study was performed on paraffin-embedded skin biopsies of normal skin (five), parapsoriasis (six), mycosis fungoides (ten patients at stage Ib and IIb) and sezary syndrome (nine). In a second part, the expression of TLRs was studied by immunohistochemistry on cutaneous section of CTCL patients after treatment with Puvatherapy and Interferon alpha. This study was performed on paraffin-embedded skin biopsies of mycosis fungoides (seven patients at stage Ib and IIb) and sezary syndrome (eight).Before treatment:

in parapsoriasis skin, TLR 2, 4 and 9 were expressed at levels similar to that observed in normal skin. By contrast, in MF skin we observed a strong intensity of labelling with the three TLRs, therefore there was no difference according the stage of MF, paradoxically this intensity decreased on SS skin.

Our results suggest two hypotheses:

Firstly, we can suppose that TLRs are internalised in keratinocytes in response to overstimulation in SS skin.

Secondly, a default of TLRs expression may exist in SS skin. This default could contribute on SS development and therefore explain the infections sensibility (staphylococcus, herpes) in these patients.

After treatment with puvatherapy and interferon alpha: in MF skin, TLRs 2, 4 and 9 were expressed at lower levels to that observed before treatment. TLRs expression in MF skin was inhibited by treatment. In contrast, in SS skin the three TLRs were expressed at similar levels to that observed before treatment.

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TNFR1- and IFN-γ-Signaling Determine whether T Cells Promote or Arrest Multistage Carcinogenesis

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The reprogramming of somatic cells during tumorigenesis leads to expression of new tumor associated antigens (TAA) that can elicit specific T cell responses. Even though cytolytic $CD8^+$ T cells (CTL) are considered to be important in anti-tumor immunity, recent data show that adaptive immune responses may control tumor growth also through mechanisms different from CTL-mediated killing or direct tumor cell lysis. In addition, independent studies unraveled that TAA-specific, IFN-γ-producing $CD4^+$ T cells prevent growth and development of transplanted tumors more efficiently than CTL. As the pro-inflammatory *in vivo* functions of Th1 cells strictly depend on the coordinated interaction of two cytokines, IFN-γ and TNF, we investigated the effects of IFN-γ- and TNFR1-signaling on multistage carcinogenesis, in RIP1-Tag2-mice expressing oncogenic T antigen (Tag) in pancreatic islets. We generated Tag-specific Th1 cells *in vitro* by stimulating $CD4^+$ cells from Tag-T cell receptor (TCR) transgenic C3H mice. Starting week 7, the time when islets have transformed into adenomas, we treated RIP1-Tag2-mice with 10^7 Tag-Th1 cells weekly. Tag-Th1 cells enriched selectively inside the Tag-expressing pancreas and tumor-draining lymph node. Through combined TNFR1- and IFN-γ-signaling, Tag-Th1 cells arrested multistage carcinogenesis, induced anti-angiogenic chemokines and prevented $\alpha_v\beta_3$ integrin-expressing tumor vessels. Arresting multistage carcinogenesis was independent of $CD8^+$ cells or significant destruction of Tag-expressing islet cells. Surprisingly, in $TNFR1^{0/0}$xRip1-Tag2 mice the same T cells paradoxically accelerated cancer development and progression. Similarly, inactivating IFN-γ with mAb did not only abolish the therapeutic effect of Tag-Th1 cells. While decline of blood glucose and death occurred as rapidly as in untreated mice, tumor growth and angiogenesis were at least two times more pronounced than in untreated mice. Thus, tumor-specific T cells can not only kill tumor cells, tumor vessels or tumor stroma, the data provided here strongly suggest that they directly survey multistage carcinogenesis through cytokine-signaling.

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Immunohistochemical Localization of Cathepsin L and Cystatin A in Normal Skin and Skin Tumors

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Cathepsin L, a cysteine proteinase, and cystatin A, an inhibitor of cysteine proteinases, are thought to regulate the invasion and metastasis of malignant cells. In this study, the expression of cathepsin L and cystatin A in skin tumors was investigated immunohistochemically in order to examine the relationship between these two enzymes in the pathophysiology of malignant cells. Formalin-fixed and paraffin embedded specimens from normal skin, seborrheic keratoses, and squamous cell carcinomas were reacted with polyclonal antibodies against rat cathepsin L or cystatin A which cross-react to human cathepsin L and cystain A, respectively. The consequent immmunostaining of these enzymes was observed to be strong in normal skin (4 cases) and seborrheic keratosis (6 cases). In well-differentiated squamous cell carcinoma (SCC) (9 cases), staining of cathepsin L and cystatin A was moderately positive in differentiated tumor cells and negative in undifferentiated SCC (5 cases). The degree of staining of these enzymes was inversely correlated with the differentiation of the malignant cells. These results suggest that the immunohistochemical analysis of cathepsin L and cystatin A is a useful indicator for an aspect of malignancy in human epidermal keratinocytes.

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Connexins 26 and 43 are Differentially Induced in Skin Tumors and the Adjacent Epidermis

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Gap Junctions have been shown to play a role in tumor progression including a variety of keratinocyte-derived and non-keratinocyte-derived skin tumors. We here show that the synthesis of the gap junction proteins Connexin 26 and 30 (Cx26 and Cx30) is induced in keratinocyte-derived epithelial skin tumors while there is either no change or a down-regulation of Cx43. Cx26, Cx30 and Cx43 are absent in non-epithelial skin tumors. Further, Cx26 and Cx30 are induced in the epidermis adjacent to malignant melanoma but absent in the epidermis adjacent to benign non-epithelial skin tumors (melanocytic nevi and angioma). The keratinocyte-derived skin tumors are very heterogeneous concerning the Cx26/Cx30 pattern in the epidermis at the periphery of the tumors. We did not observe any difference in the localization of the very similar proteins Cx26 and Cx30 but a variation in intensity of immunoreactivity. As the staining patterns of Cx26 and Cx30 antibodies are not identical with those of CK6, a marker of hyperproliferation, and CK17, a marker for trauma, we discuss that the induction of these gap junctional proteins exceeds reflection of reactive hyperproliferative or traumatized epidermis. We further discuss the putative roles of these gap junctional proteins in tumor progression.

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The Steroid Receptor Co-Activator-1 (SRC-1) Potentiates TGF-beta/Smad Signaling by Enhancing Smad3-p300/CBP Interactions

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The three related 160-kDa proteins, SRC1, TIF2, and RAC3, which form the p160 family of coactivators, were initially identified as factors interacting with nuclear receptors (NRs). They have also been reported to potentiate the activity of a number of other transcription factors, including AP-1 and NF-kappaB. The aim of this work was to identify whether SRC-1 also interferes with the TGF-beta/Smad signaling pathway, and if so, to identify its underlying mechanisms of action. Transient cell transfection experiments performed in human dermal fibroblasts using the artificial Smad3/4-specific (SBE)4-lux reporter construct, as well as p800-lux, which harbors 800 bp of the human PAI-1 promoter driving the expression of luciferase, we determined that overexpression of SRC-1 enhances TGF-beta-induced, Smad-mediated, transcription. Likewise, SRC-1 overexpression potentiated TGF-beta-induced upregulation of *PAI-1* steady-state mRNA levels. Using a mammalian two-hybrid system approach, we demonstrated that SRC-1 interacts with the trasncriptional co-activators p300/CBP, but not with Smad3. Overexpression of the adenovirus E1A oncoprotein, an inhibitor of CBP/p300 activity, prevented the enhancing effect of SRC-1 on Smad3/4-mediated transcription, indicating that p300/CBP may be required for SRC-1 effect. Indeed, such hypothesis was validated, as overexpression of a mutant form of SRC-1 lacking the CBP/p300-binding site failed to upregulate Smad3/4-dependent transcription. In addition, using a mammalian two-hybrid approach, we found that SRC-1 potentiates p300 Smad3 interactions while not binding Smad3 directly. Together, these results identify SRC-1 as a novel Smad3/4 transcriptional partner facilitating the functional link between Smad3 and p300/CBP, resulting in the enhancement of TGF-beta/Smad3-mediated transcription.

341 [Oral 022]

HB-EGF Shedding is Essential for UV-Induced EGFR Phosphorylation and Epidermal Hyperplasia

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UV irradiation has various biological effects on skin. One is EGF receptor (EGFR) phosphorylation and the subsequent activation of MAP kinase, leading to epidermal hyperplasia. However, the cross-talk mechanism linking UV irradiation and EGFR phosphorylation remains unclear. Recently, it was reported that EGFR transactivation by G-coupled-protein receptors is mediated via HB-EGF shedding, the conversion of HB-EGF from a membrane anchored form to a soluble form. So we hypothesized that UV-induced EGFR phosphorylation is mediated via HB-EGF shedding. First we examined the effect of UV irradiation on EGFR phosphorylation in human keratinocytes. UV irradiation (30 mJ/cm^2) induced EGFR phosphorylation in a time dependent manner, optimally 2.1 fold at 20 min. Next we examined whether UV irradiation induces HB-EGF shedding from the keratinocyte cell surface. Biotinylated membrane-anchored HB-EGF on the cell surface disappeared almost completely 10 min after UV irradiation. The soluble form of EGFR ligands in culture medium was measured by bioassay. Soluble EGFR ligands increased optimally 1.5 fold at 5 min after UV irradiation. An inhibition assay identified 77% of the soluble EGFR ligands as HB-EGF. Furthermore, R8301, a specific inhibitor of HB-EGF shedding, inhibited UV-induced HB-EGF shedding completely at 1 μM. We also confirmed the HB-EGF inhibitors such as R8301, anti-HB-EGF blocking antibody and CRM197, a specific inhibitor of soluble HB-EGF, completely inhibited UV-induced EGFR phosphorylation. Finally, we examined the involvement of HB-EGF in UV-induced epidermal hyperplasia using keratinocyte-specific HB-EGF knockout mice generated by Cre/loxP technology. In HB-EGF knockout mice, UV-induced epidermal hyperplasia was completely inhibited. In conclusion, we clearly demonstrated that UV irradiation-induced EGFR phosphorylation and epidermal hyperplasia are mediated via HB-EGF shedding, the conversion of HB-EGF from a membrane-anchored form to a soluble form.

342 [Oral 035]

Keratin5-Cre/LoxP Mediated Deletion of Vascular Endothelial Growth Factor Severely Compromises Mammary Gland Function in Association with Reduced Blood Vessel Density and Duct Development

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In the adult, the process of blood vessel formation, or angiogenesis, is restricted to settings of increased vascular requirements, such as wound healing, tumor growth and the female reproductive tract during pregnancy and lactation. Expression of the blood vessel endothelial cell mitogen, Vascular Endothelial Growth Factor (VEGF), is upregulated in keratinocytes during wound healing and epidermal tumors, and has been shown to be regulated in concert with mammary gland development during pregnancy and lactation, suggesting that this factor is important for angiogenesis in these settings. Using the Cre/LoxP system under the control of the keratin5 promoter, we have generated mice in which VEGF has been inactivated in all keratin5 expressing tissues, including the female reproductive tract. We have shown previously that these mutant mice (K5/Cre VEGF F/F), in addition to impaired wound healing and tumor formation, display severely compromised mammary gland function: they were unable to adequately nourish their young, and mammary gland duct development was reduced during pregnancy and lactation. We now report that this retarded development in the mutant mammary glands during pregnancy and lactation is associated with a failure to upregulate production of VEGF, and with impaired angiogenesis. Histomorphometric analysis revealed that blood vessels occupied a significantly smaller area in mutant mice and appeared thinner and less well organized than those of controls. The composition of the milk itself showed no differences in the major protein components, nor was there a difference in casein content as judged by silver staining and Western blotting respectively. We conclude, firstly, that the mammary gland epithelial cells, and not stromal cells, are the major source of VEGF during pregnancy and lactation. Secondly, in the absence of epithelial cell derived VEGF, mammary gland duct and blood vessel proliferation is reduced during these processes, resulting in reduced milk production, and inadequately nourished offspring.

343 [Oral 034]

Simultaneous Inhibition of VEGFR1 and VEGFR2 Signaling is Required for Suppression of Experimental Melanoma Metastases

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The propensity of tumor cells to colonize remote organs is greatly affected by active tumor-host interactions. Accumulating evidence suggests that the host microenvironment is also influenced by bone marrow-derived hematopoietic and endothelial stem/progenitor cells, prone to collaborate on promoting tumor cell survival and neovessel-dependent tumor growth. As metastasis continues to be the major cause of morbidity and mortality in malignancies, we sought to extend the understanding about the role of vascular endothelial growth factor-positive (VEGFR+) bone marrow-derived progenitors in malignancies by employing an experimental melanoma metastasis assay. Our studies reveal that experimental metastasis formation mobilizes both VEGFR1+ myloid cells and VEGFR2+ endothelial progenitors. In transplantation studies utilizing genetically marked bone marrow, however, examination of lung metastases showed recruitment primarily of myelomonocytic and megakaryocytic bone marrow-derived cells, whereas endothelial progenitors failed to considerably integrate in metastases. We therefore explored whether inhibition of VEGFR1 or VEGFR2 signaling leads to distinct suppressive effects on metastasis formation. Remarkably however, only simultaneous antibody-mediated inhibition of VEGFR1 and VEGFR2 signaling suppressed lung metastasis formation. While our data suggest that the anti-metastatic effects achieved by combined inhibition of VEGFR1 and VEGFR2 signaling are mediated via targeting cell populations other than bone marrow-derived progenitors, we herein provide first evidence that combined anti-VEGFR1 and anti-VEGFR2 strategies could potentially open up further prospects to interfere with metastatic growth in secondary organs.

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Convergence of the p38/ERK/MSK1 and SMAD Signaling Pathways in the Induction of c-fos Gene Expression by Interleukin-1β and TGF-β

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Interleukin-1β (IL-1β) plays a central role in acute and chronic inflammation. At the transcriptional level, IL-1β promotes expression of a variety of immediate-early-response genes like *c-fos*. In turn, immediate-early-response genes influence the expression of secondary response genes contributing to the phenotypic response of the cell to proinflammatory stimuli. It is known that stimulus-dependent gene transcription of *c-fos*, is mediated via several *cis*-acting elements, including at least three cAMP response elements and a serum response element. However, the precise mechanisms by which IL-1β induce *c-fos* gene expression remain unclear. Here we show that inhibition of either ERK or p38 mitogen-activated protein kinases (MAPK) abolished IL-1β-induced *c-fos* gene expression in human HaCaT keratinocytes as revealed by northern blotting and by use of a c-fos-promoter-containing reporter construct. We further provide evidence that the p38-MAPK- and ERK-activated kinase MSK-1 is required for full *c-fos* gene transcription, as well as for the phosphorylation of cAMP response element-binding protein (CREB). In addition, we demonstrate that IL-1β-induced c-fos gene transcription is potentiated by TGF-β through a mechanism involving SMAD activation. In conclusion these results show that MSK1 and downstream CREB-phosphorylation are important p38-MAPK- and ERK-activated mediators of IL-1β-stimulated c-fos induction. In addition, these studies provide evidence that following IL-1β and TGF-β stimuli, both the p38/ERK/MSK1 and the Smad pathways converge to the regulation of the prototypical immediate-early-response gene *c-fos*.

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TGF-beta and TNF-alpha: Antagonistic Cytokines Controlling Connexin 43 Gene Expression

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Among the structures mediating cell-cell interactions, gap junctional intercellular communications (GJIC) are unique in that cells directly transfer small water-soluble molecules ($Mr < 1000$) from the inside of one cell to that of neighboring cells. Gap junctional channels are composed of two connexons, each composed of six subunit proteins termed connexins (Cx). A wealth of independent observations implies that changes in the proliferation program of keratinocytes coincide with a dynamically regulated switch of the pattern of Cx expression in the skin. The aim of this work was to examine the effect of two antagonistic cytokines implicated in the control of keratynocyte proliferation, TGF-beta and TNF-alpha, on the expression the connexin43, the most abundant Cx in human epidermis, not only expressed throughout the spinous and granular cell layers, but also focally in the basal epidermal layer. Using transient cell transfections of the human keratinocyte cell line HaCat with Cx43 promoter/reporter gene constructs, we determined that TGF-beta increases Cx43 transcription whereas TNF-alpha represses Cx43 promoter activity. The physiological relevance of these observations was confirmed as modulation of Cx43 steady-state mRNA and protein levels by these cytokines paralleled that of the Cx43 promoter. Next, using expression vectors for either a dominant-negative form of Smad3 or for antisense cJun and/or JunB mRNA, we demonstrated that enhancement of Cx43 transcription by TGF-beta requires both Smad3/4 and Jun/AP-1 signaling pathways. On the other hand, using either a pharmacological approach or overexpression of dominant-negative upstream kinases to inhibit the JNK, ERK and p38 MAP kinase pathways, we identified both JNK and p38 as critical for decreased Cx43 transcription by TNF-alpha. Further studies are required to determine the link between these regulatory pathways, Cx43 expression, and the control of keratinocyte proliferation by TGF-beta and TNF-alpha.

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Impaired Wound Contraction in Murine Leukocyte-Adhesion Deficiency 1 Relies on Decreased TGF-β_1 Secretion and Impaired Differentiation of Myofibroblasts

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The β_2 leukocyte integrins are heterodimers, composed of a common β chain (CD18) and one out of four distinct α chains (CD11). They are pivotal for migration and signalling of hematopoietic cells during inflammatory processes and immune responses. Lack of functional β_2 integrin causes leukocyte-adhesion deficiency type 1 (LAD1), a life-threatening primary immunodeficiency syndrome with severe recurrent microbial infections, leukocytosis and impaired wound healing.

Initial data revealed significantly increased wound sizes in a murine model for LAD1 ($CD18^{-/-}$) from day 5 to 14 after application of full thickness wounds on mouse backs. We now addressed the question whether this impairment in wound healing was a consequence of reduced wound contraction potentially caused by disturbed myofibroblast recruitment. Therefore, we analyzed expression of markers critical for myofibroblast differentiation by immunohistochemistry and Western blotting. We found that both splice variant ED-A of fibronectin and α-smooth muscle actin were substantially reduced in $CD18^{-/-}$ mice at day 5 and 7 after wounding, respectively, indicating impaired myofibroblast differentiation. Interestingly, TGF-β1 and its receptor TGF-βRII were also largely decreased. Since TGF-β_1 is a key factor for granulation tissue formation and promotes wound contraction, we supplemented TGF-β_1 by subcutaneously injecting two different doses (0.45 μg, 0.1 μg) into the wound margins at day 1, 3 and 5 after wounding of $CD18^{-/-}$ and wild-type mice. As a result, we observed a rescued wound closure in $CD18^{-/-}$, similar to wild-type mice.

Considering the essential requirement for CD18 in neutrophil extravasation and migration into wound tissue, we hypothesize that due to the absence of neutrophils in wounds of $CD18^{-/-}$ mice, macrophages were not able to phagocytose apoptotic neutrophils and thus, they lacked the critical stimulus to secrete TGF-β_1. Deviant from former views, our data demonstrate that growth factors released by neutrophils in a paracrine fashion are important for wound contraction and healing. It remains to be seen whether CD18 is also involved in migration of myofibroblast precursors.

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Small Rho GTP Binding Proteins Regulate Vascular Endothelial Growth Factor-2 Expresssion In Endothelial Cells

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Recent evidence suggests, that rho-family small GTPases play a significant role in the regulation of different endothelial cell functions, implicated in capillary network formation. Whereas Rho-family small GTPases are primarily known to control actin-based motility processes, RhoA, Rac1 and Cdc42 have been shown to regulate other cellular activities as well, such as membrane transport and gene transcription. As vascular endothelial growth factor receptor-2 (VEGFR2) expression is necessary for angiogenic responses to occur, we hypothesized that Rho-family small GTPases may affect VEGFR2 expression by cultured endothelial cells (ECs). Overexpression of dominant negative mutants N17Rac1 and N19RhoA were seen to significantly inhibit VEGFR2 protein expression by HUVEC, whereas transfection of mutant N17Cdc42 failed to affect VEGFR2 levels. As N17Rac1 and N19RhoA also supressed VEGFR2 mRNA accumulation, we subsequently examined their effects on VEGFR2 transcriptional activation. Analyses of a different 5′-deletional VEGFR2 promoter-based reporter gene constructs revealed that inhibition by N17Rac1 and N19RhoA is conveyed by distinct gene regulatory elements. Whereas N17 Rac1-mediated supression is confined to a GC-rich region between bp -77 and -60, N19RhoA-mediated inhibition appears to be conferred by an element located between bp -225 and -160, harboring a consensus E-box binding site. We were able to show by EMSA analysis, that constitutive Sp1-dependent DNA binding to the GG-rich region is decreased by N17Rac1 transfection, indicating that inhibition of rac1 may interfere with Sp1-dependent VEGFR2 transcription. Therefore, different members of the Rho-family small GTPases exert diverse effects on VEGFR2 expression. Significantly, inhibition of VEGFR2 transcription by N17Rac1 and N19RhoA involves distinct molecular mechanisms as different elements seem to be engaged. In conclusion, control of capillary network formation by Rho-family small GTPases may likely be mediated in part via modulation of VEGFR2 expression.

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BDNF upregulates TGFβ2 and Induces Keratinocyte Apoptosis and Regression of Cultured Human Anagen Hair Follicles

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Neurotrophins are important modulators of epithelial-mesenchymal interactions. Previously, we had shown that brain derived neurotrophic factor (BDNF) and its high affinity receptor (TrkB) are prominently involved in the control of murine hair follicle cycling (FASEB J 13: 395, 1999). We now show that BDNF and TrkB are also expressed in the human hair follicle in a manner that is both hair cycle-dependent and suggestive of epithelial-mesenchymal cross-talk between BDNF secreting dermal papilla fibroblasts of anagen hair follicles and subpopulations of TrkB+ hair follicle keratinocytes. As first functional evidence for an involvement of BDNF/TrkB in human hair growth control, we show that 50 ng/ml BDNF significantly inhibits hair shaft elongation in organ-cultured human anagen hair follicles and induces premature catagen development. Hair follicle regression is associated with induction of keratinocyte apoptosis – as evidenced by nick-end-labeling – and inhibition of keratinocyte proliferation. Immunohistochemistry and quantitative realtime rtPCR analysis demonstrates up-regulation of the potent catagen inducer, tumor growth factor β2 (TGFβ2) by BDNF. Catagen induction by BDNF was partially reversible through co-administration of TGFβ-neutralizing antibody, and this treatment downregulated TGFβ2-expression in the hair bulb by immunohistochemistry. These findings suggest, that TrkB-mediated-signaling promotes the switch between anagen and catagen at least in part via up-regulation of TGFβ2. Thus, human scalp hair follicles are both a source and target of bioregulation by BDNF, which invites to target TrkB-mediated signaling for therapeutic hair growth modulation.

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Nerve Growth Factor Regulates Hair Cycle Progression in Humans and Mice

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Neurotrophins are more than nerve growth factors. Ample evidence proves, e.g. that the prototypic nerve growth factor (NGF) promotes proliferation of keratinocytes via its high affinity receptor tyrosine kinase A (TrkA). In contrast, its precursor pro-NGF, which is spliced from the same mRNA promotes apoptosis via p75, a member of the tumor necrosis factor receptor family. p75 has a low affinity for NGF and a high affinity for pro-NGF. Interestingly, pro-NGF is contained in all commercially available NGF-preparations. Recently, we were also able to demonstrate a role for NGF in stress-triggered hair growth termination. Here we show, that NGF and its receptors TrkA and p75 are expressed in human and murine hair follicles with distinct expression patterns in respect to anatomical structure and hair cycle stage. E.g. by mRNA pro-NGF/NGF level peaks in early anagen, dramatically decreases towards high anagen and recovers in catagen while by Elisa, pro-NGF/NGF protein peaks in catagen. By immunohistochemistry, NGF and TrkA are found in the outer root sheath throughout the hair cycle in man and mice with the strongest expression in the proliferative basal layer. Strong pro-NGF expression is found in the inner root sheath and p75 is detectable in the dermal papilla of late anagen hair follicles prior to the onset of catagen and in the regressing epithelial strand of human and murine catagen hair follicles. These distribution patterns suggest a role for NGF/TrkA interactions in follicular keratinocyte proliferation and hair growth as well as a role for pro-NGF/p75 interactions in keratinocyte apoptosis and hair follicle regression. In accordance with these data we could show that NGF promoted anagen in organ cultured murine early anagen hair follicles. In contrast, catagen was induced by NGF in organ cultured human and murine late anagen hair follicles. We thus conclude, that NGF live and death decisions in hair growth control depend on NGF-processing and availability of high and low affinity receptors. The development of NGF-preparations exclusively containing NGF or pro-NGF may provide a promising strategy for the development of new therapeutic approaches to treat hair growth disorders.

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The Changing Role of TGFβ1 During Wound Repair

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Healing of cutaneous wounds proceeds via a fibro-proliferative process. Fibroblasts migrate into the wound, proliferate, differentiate into myofibroblasts, and synthesise ECM proteins, forming early granulation tissue. This first phase of wound repair is influenced by the TGFβ family of pleiotropic factors. Nevertheless TGF-β1 levels remain high well beyond the completion of the fibroproliferative phase, suggestive of a further role. On transition of late stage granulation tissue into mature scar, redundant cells are eliminated from the maturing tissue via apoptosis (induction mechanism unknown). It is possible that TGF-β also modulates the remodelling/apoptosis phase of wound repair as it is an effector of apoptosis in a variety of cell types. We hypothesise that TGF-β1 modulates the entire wound healing process. The pleiotropic roles being directed by the cell type present, cell density, matrix environment, integrins, enzymes and peptides present at any given time. To investigate this theory we embedded dermal fibroblasts derived from normal skin ($n = 5$) into a variety of growth environments simulating the different phases of wound healing and studied the effects of different doses of TGF-β1 in relation to cell proliferation, morphology, differentiation and apoptosis. Fibroblasts cultured in anchored fibrin and collagen gels showed completely different morphology after treatment with TGF-β1: cells in fibrin exhibited a striking increase in cell–cell contact. Induction of myofibroblasts differentiation by TGFβ was equivalent in both matrices. Surprisingly, we found TGF-β1 at 2 ng/ml significantly ($p < 0.001$) induced apoptosis (>50%) of fibroblasts embedded in 3-D collagen matrices irrespective of whether the cells were allowed to contract/remodel the matrix or not. No apoptosis was detected in 3-D fibrin matrices. Higher concentrations of TGF-β1 had a mitogenic effect (>8 ng/ml). This apoptosis-inducing effect of TGF-β1 was reliant on the three dimensional nature of the collagen gels and not simply integrin binding to collagen as TGF-β1-treatment of cell monolayers cultured on collagen-coated plastic did not elicit an apoptotic response. These results suggest for the first time a potential role for the involvement of TGF-β1 in the induction of apoptosis during wound repair.

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Heparin-Binding EGF (HB-EGF) Induces the Pro-Angiogenic VEGF-Coreceptor Neuropilin-1 in HaCaT Keratinocytes by Downregulating the Repressor Transcription Factor NRSF (Neural-Restrictive Silencing Factor)

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Neuropilin-1 (NRP-1), a cell surface receptor for secreted semaphorins as well as for VEGF165, has been shown to be necessary for both developmental and tumor angiogenesis. The regulation of neuropilin-1 is only poorly understood. Here we show evidence that a consensus binding sequence in the neuropilin-1 promoter known as a neural restrictive silencer element (NRSE) is implicated in the transcriptional repression of NRP-1. This binding site is recognized by the neural restrictive silencing factor NRSF, also known as RE-1 silencing transcription factor (REST). In HaCaT keratinocytes mutation of the NRSE leads to increased promoter activity, as shown by luciferase reporter gene assays. By electromobility shift assays it could be demonstrated that NRSF protein binds to NRSE in the NRP-1 promoter. HaCaT keratinocytes stably overexpressing NRSF had decreased NRP-1 levels, whereas blocking of NRSF activity by a truncated, dominant-negative NRSF protein caused derepression of NRP-1. Similar results were obtained when cells were treated with the histone deacetylase inhibitor trichostatin A, an inhibitor of NRSF silencing activity. Furthermore, when HaCaT cells were stimulated with HB-EGF, a strong inducer of NRP-1 expression, increase of NRP-1 gene expression was accompanied by a decrease in NRSF mRNA and protein levels. Taken together, these results indicate that NRP-1 expression is negatively regulated by NRSF. It is concluded that despite its main function of repressing neuronal genes in non-neuronal cells NRSF might have an important role in the regulation of vascular development and angiogenesis, representing the first known negative regulator of the isoform-specific VEGF-coreceptor neuropilin-1 in a physiological context.

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Evidence for Expression of Multiple Members of the Family of Suppressors of Cytokine Signalling in Normal and Transformed Human Melanocytes

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Suppressors of cytokine signalling (SOCS) define a newly identified family of negative regulators of cytokine signalling. At present, this family consists of 8 structurally related members, SOCS-1 to -7, and CIS. We recently observed that SOCS-1 is a progression marker of human melanoma. Here we investigated the expression of additional members of the SOCS family in normal human melanocytes (NHM) and 8 human melanoma cell lines by multi-probe ribonuclease protection assay. CIS, SOCS-5 and -7 were similarly expressed in all of the melanoma cell lines and NHM. RNA levels of SOCS-6 were significantly higher in NHM compared with all melanoma cell lines. Most interestingly, SOCS-3 expression was silenced in NHM. In most melanoma cell lines (6 out of 8) SOCS-3 was highly expressed while in 2 melanoma cell lines SOCS-3 expression was marginal. To assess the relevance of these findings, the *in vitro* interferon (IFN-alpha) and interleukin 6 (IL-6) sensitivities of the various melanoma cell lines were determined by proliferation assays. There was no correlation between the IFN-alpha sensitivity and SOCS-3 expression. On the other hand, the IL-6 sensitivity closely correlated with SOCS-3 mRNA expression. At the protein level SOCS-3 expression was absent in NHM as shown by Western immunoblotting. In melanoma cell lines SOCS-3 protein expression correlated with expression of Pim-2, a serine/threonine kinase previously implicated in stability and phosphorylation of the SOCS-1 protein. In summary, our findings, for the first time, show that multiple members of the SOCS family are expressed in human pigment cells. SOCS-3 gene expression tightly correlates with IL-6 sensitivity in melanoma cells *in vitro*. Further studies are needed to determine if SOCS-3 RNA knock-out can restore IL-6 sensitivity, and if SOCS-3 is expressed in melanoma *in situ*.

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NFκB Selectively Enhances UVB-Induced Apoptosis Via Differential Gene Regulation

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Activation of the transcription factor nuclear factor-κB (NFκB) by interleukin 1 (IL-1) is generally associated with the induction of antiapoptotic pathways within the cell. Accordingly, NFκB was shown to suppress death ligand-induced apoptosis through transcriptional up-regulation of antiapoptotic proteins IAPs (inhibitor of apoptosis proteins) and FLIP (I-FLICE). In contrast, we could demonstrate that UVB-mediated cell death was significantly enhanced upon NFκB activation. Enhancement of UVB-induced apoptosis coincided with a release of about 100 pg/ml of the proapoptotic cytokine TNFα. Surprisingly, co-stimulation of cells with the same dose of TNFα and UVB or application of supernatants of IL-1plus UVB-treated cells to UVB-irradiated cells resulted only in a rather weak enhancement of UVB-induced apoptosis, indicating that other intracellular components have to be involved in the proapoptotic effect of NFκB. Gene chip arrays revealed that, in contrast to the NFκB-dependent transcriptional up-regulation of TNFα, NFκB-dependent transcription of c-IAP and FLIP was completely inhibited by UVB radiation. In addition, members of the TRAF (tumor necrosis factor receptor-associated factor) protein family which contain NFκB consensus elements within their promoters were shown to be significantly down-regulated upon UVB exposure. TRAF proteins are upstream adapter proteins associated with the cell death receptor TNFR-1. Upon activation by TNFα TNFR-1 can transduce either pro- or antiapoptotic signals depending on the distribution of certain adapter proteins. Whereas recruitment of the proapoptotic adapter protein FADD results in induction of apoptotic cell death, the recruitment of TRAF proteins activate antiapoptotic signalling pathways mediating cell survival. Conclusively the downregulation of TRAF proteins results in promotion of the proapoptotic pathway triggered by TNFR-1, thereby enhancing UVB-induced apoptosis. Taken together, NFκB activation in association with UVB exposure results in up-regulation of the proapoptotic death ligand TNFα. In addition, it results in transcriptional inhibition of antiapoptotic cIAP and FLIP genes and of TNFR-1 related antiapoptotic adapter proteins TRAFs. In contrast to the general assumption, NFκB in combination with UVB radiation has the potential to act in a proapoptotic fashion.

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Blockade of β_1 Integrin Activates Caspase-8 Apoptotic Pathway without Fas/FasL Modulation in Human Keratinocytes

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Anoikis is a mode of cell death that occurs after detachment from the extracellular matrix. We have previously shown that transit amplifying cells, but not keratinocyte stem cells undergo anoikis after disruption of β_1 integrin signal. We intended to further evaluate the mechanism underlying this phenomenon. Normal human keratinocytes were cultured from neonatal foreskin and either left in suspension or provided with anti-β_1 integrin neutralizing antibody. We showed by TUNEL technique that the addition of anti-β_1 integrin significantly accelerates anoikis in keratinocytes, as compared to merely suspended cells. In order to dissect which apoptotic pathway is involved in keratinocyte anoikis, we evaluated the hierarchical activation of caspases after treatment with anti-β_1 integrin. Caspase-8 activation and cytochrome c release from mitochondria were observed at 3 hrs, while caspase-9 was activated at 6 hrs. Caspase-9 inhibitor zLEDH-fmk failed to block caspase-8, -10, -3 and Bid activation. On the contrary, caspase-8 inhibitor zIETD-fmk delayed the activation of caspase-9 and -3, and blocked the activation of Bid. Anti-β_1 integrin failed to cleave Bid, to release cytochrome c and to activate caspase-9 in c-FLIP (FLICE/caspase-8 inhibitory protein) overexpressing keratinocytes. Finally, anti β_1 integrin induced anoikis in c-FLIP transfected keratinocytes to a lesser degree, as compared to mock cells ($p < 0.01$). These results seem to indicate that the caspase-8 activated (extrinsic) apoptotic pathway is the prevailing mechanism in anoikis. As the extrinsic apoptotic pathway is triggered by death receptor signal and anoikis has been shown to be dependent from Fas/Fas ligand system, we evaluated the expression of Fas and Fas ligand in keratinocytes after addition of anti-β_1 integrin. Blockade of β_1 integrin failed to up-regulate either Fas or Fas ligand in keratinocytes, as shown by flow cytometry. These results suggest that blocking β_1 integrin activates caspase-8 pathway without the involvement of Fas/FasL.

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IgE-Dependent Signalling in Human Basophils is Dependent on PLC-mediated p38 MAPK Activation but not Affected by cJun N-Terminal Kinase

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In allergic diseases, such as atopic dermatitis, basophils migrate into affected tissues where they secrete both inflammatory (e.g. histamine, LTC-4) and immunomodulatory mediators (e.g. IL-4, IL-13) upon contact with allergen. To date, the intracellular signals responsible for controlling the IgE-dependent release of the above mediators are poorly understood which has stifled attempts at developing pharmacological strategies that inhibit their function. In mast cells, which in some respects are tissue-fixed compatriots of basophils, IgE-dependent activation crucially involves phosphilipase C (PLC), protein kinase C (PKC) and the phosphorylation of cJun N-terminal kinase (JNK). Here, we characterised the involvement of the above signalling components in basophils following IgE-receptor activation. Basophils were obtained from healthy blood donors and purified to over 95% purity by a three-step procedure involving Ficoll-density centrifugation, elutriation and magnetic cell sorting. The activities of various MAPK enzymes following stimulation with anti-IgE for various periods were assessed by Western blotting. The results showed that, while p38 MAPK was rapidly phosphorylated in basophils, together with a slower onset of ERK1&2 activation, JNK and its downstream transcription factor cJun were not activated in the cells. Additionally, use of specific antagonists confirmed that, while p38 MAPK controls the production of all basophil mediator types and ERK1&2 only LTC-4 secretion, JNK is not involved in mediator release. Further, we observed that p38 MAPK activation is completely abolished by PLC inhibitors while it is less affected by specific PKC antagonism. We conclude that p38 MAPK is the most important MAPK family member involved in IgE-dependent signalling in basophils and is regulated by PLC, and subsequent calcium mobilization, but not by PKC. The data also highlights major differences between basophils and mast cells regarding the control of mediator secretion by MAPK.

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Insulin-like Growth Factor-I (IGF-I) Enhances Invasive Activity of Malignant Triton Tumor Cells

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Malignant triton tumor (MTT) is a rare variant of malignant peripheral nerve sheath tumor (MPNST) with rhabdomyoblastic change. This rare tumor shows the poorer prognosis than MPNST. Five year survival rate has been reported to be 3–10% in MTT whereas it is 50–60% in MPNST. The malignant behavior of MTT cells has not yet been evaluated *in vitro* presumably because of its rarity. In this study, we evaluated the aggressive invasion of MTT cells into extracellular matrix and the effect of some growth factors on their invasive activity. Invasive assay using modified Boyden chamber showed the higher invasive activity of MTT cells than neurofibroma cells, fibroblasts and dermatofibrosarcoma cells. The aggressive invasion of MTT cells was enhanced by IGF-I but not by epidermal growth factor, basic fibroblast growth factor and hepatocyte growth factor. In accordance to the finding, IGF-I augmented α3 integrin subunit expression on MTT cell surface and enhanced attachment of MTT cells to type I collagen, fibronectin and laminin. The induction of α3 integrin expression attained a maximal level at a concentration of 10 ng/ml and after 36 h exposure of IGF-I. The results suggest the contribution of IGF-I to augmentation of invasive and metastatic potential of MTT cells and poor prognosis in MTT patients.

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Wound Healing and Skin Barrier Repair are Unaffected in IL-15 Deficient Mice

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Wound repair, the restoration of permeability barrier against excessive water loss and the entry of pathogenic microbes after injury, is an essential for survival. Wound healing is a complex process which involves different cell types, cytokines, and growth factors. It has been demonstrated that γδ-dendritic epidermal T-cells (DETC) are important to ensure a correct growth factor environment for wound healing and provide KGF for keratinocyte proliferation; DETCs need IL-15 to survive. The IL-15 receptor (IL-15 R) consist of three subunits, the IL-15 α-chain, IL-2β-chain and the IL-2 γ_c-common chain. Deletions in the IL-15, IL-15 Rα or IL-2β locus lead to an impaired development of γδ-T cells in epithelia (Ye *et al*, 2001; Suzuki *et al*, 1997; Lodolce *et al*, 1998). Transplanted γδ-DETC were not able to survive in the skin of IL-15 knock out mice. It has been described that mice lacking γδ-DETC in the skin showed a delayed wound repair. We investigated the effect of IL-15 deficiency in mice on the repair of superficial and full thickness skin wounds. Superficial wounds were induced by tape-stripping and the repair rate was determined by the recovery in transepidermal water loss as a marker of barrier function. The repair rate in full thickness wounds 10 mm in diameter on the flank of the mice was determined by measurements of the wound size using a computerized digital imaging system. In addition, wound contraction and reepithelization was evaluated. The repair rate in superficial wounds of IL-15 deficient mice did not show significant differences compared to wild type mice. Also, the healing rate in full thickness wounds, neither overall wound size nor wound contraction or reepithelization were different in deficient compared to wild type mice. Our results suggest that alternative pathway to set the required cytokine environment for wound healing. The function of γδ-DETC may be compensated by other mechanisms.

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Reduced Reepithelialisation of Human Neoepidermis by a Small-molecular Epidermal Growth Factor Receptor Inhibitor

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Psoriasis and several epidermal tumours are characterised by abnormal keratinocyte proliferation and epidermal growth factor receptor (EGFR) overexpression. Consequently, EGFR is recognised as a potential target for psoriasis and skin cancer therapy. Although EGFR-inhibiting compounds reduce the mitotic activity of keratinocytes in monolayer culture, this class of drugs is not widely investigated in 3-D skin models or clinically in hyperproliferative or malignant dermatoses. The aim of this study was to assess the dynamic and morphological effects of EGFR inhibition on multilayered neoepidermis produced from skin explants grown on deepidermised dermis at air-liquid interface in classical medium. The small-molecular inhibitor of EGFR (HER1) and HER2 tyrosine kinases, PKI166, was applied at 0.5-5 μM for 10 days in culture. The surface area of newly formed epidermis was determined repeatedly by fluorescence imaging of reepithelialisation ('FIRE') technique and related to the effects of PKI166 on receptor autophosphorylation, tissue morphology, and BrdU labelling index. Addition of ligand (EGF) increased neoepidermal outgrowth rate and mitotic activity, whereas PKI166 decreased EGFR activation and rate of resurfacing irrespective of exogenous EGF. The dynamic effects of PKI166 were dose-dependent, and no histological signs of neoepidermal drug toxicity were observed. The study illustrated the utility of fluorescence imaging in quantifying living neoepidermis, and emphasised the potential of EGFR inhibition in decreasing outgrowth of human epidermis on deepidermised dermis. Therapeutic intervention of EGFR signalling by selective tyrosine kinase inhibitors might be a feasible approach in hyperproliferative states of the epidermis.

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Fetal Fibroblasts Respond Quicker but More Transiently in the IGF-I and TGF-β1 Intracellular Signaling Pathways

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Cutaneous wound healing in early fetuses occurs rapidly and leads to perfect regeneration of tissue architecture rather than the formation of scar tissue, which is characteristic of postnatal healing. Pro-fibrotic growth factors such as TGF-β1 have been shown to play a role in fetal wound healing. Little is yet understood regarding the intracellular signalling pathways involved in wound healing in the fetus. We aimed to study the intracellular pathways of both IGF-I and TGF-β1. Following local ethical approval we have established primary cell lines of fetal dermal fibroblasts (FDF; $<$14 week gestation n$=$5) and postnatal dermal fibroblasts (DF; $<$9 years of age n$=$5). We used immunohistochemical staining using a commercially available antibody for smooth muscle actin and established that FDF do differentiate into myofibroblasts unlike previously believed. We have also studied the intracellular signalling pathways for IGF-I and TGF-β1 using SDS-PAGE/Western Blotting and immunoprecipitation using commercially available antibodies and have shown for a number of proteins involved in these pathways e.g. p-JNK, p-Smad2/3 and pERK that fetal signalling appears quicker but more transient. As previously shown endocytosis of receptors has been suggested to slow intracellular signalling, we have therefore studied the IGF-I receptor function in FDF and preliminary work has indicated that unlike DF cells endocytosis of the IGF-IR is not required. Our work has indicated that FDF activate the IGF-I and TGFβ1 signalling pathways, but this activation is rapid and transient. FDF also differentiate into myofibroblasts but again this differentiation is rapid but transient.

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Agonist-Induced Ca^{2+} Oscillations and Ca^{2+} Entry in Keratinocytes

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Several independent studies have provided evidence of a mitogenic effect of extracellular ATP and UTP on primary keratinocytes. Two classes of extracellular nucleotide receptors have been established: the P2X family ATP gated ion channels and the metabotropic P2Y family of G-protein coupled receptors (GPCRs). Stimulation of GPCRs activates PLC-β, leading to the production of inositol 1,4,5-triphosphate (IP_3), release of $[Ca^{2+}]_i$ from intracellular stores. Depletion of the stores in turn leads to activation of store-operated channels in the plasma membrane. The relationship between agonist-induced store depletion and Ca^{2+} influx in HaCaT and normal human keratinocytes (NHKs) has not been fully characterized. Here, we show that stimulation with UTP or ATP elicited pronounced Ca^{2+} influx in HaCaT keratinocytes under low (70 μM) Ca^{2+} conditions. In contrast, cytosolic Ca^{2+} rapidly returned to resting levels in NHKs stimulated with UTP or ATP. Chelation of extracellular Ca^{2+} with EGTA indicated that modest Ca^{2+} influx occurred following the initial Ca^{2+} transient, but this was significantly less than the Ca^{2+} entry observed in HaCaTs. Only a single Ca^{2+} transient was detected in most NHKs exposed to ATP or UTP. However, in a subset of cells, a second or third Ca^{2+} transient was observed at later time points. The amplitudes of the later Ca^{2+} transients were smaller than that of the initial Ca^{2+} signal. When extracellular Ca^{2+} was raised to physiologically relevant levels (1.2 mM), stimulation with ATP or UTP resulted in prolonged Ca^{2+} entry. This was not observed in cells in which $[Ca^{2+}]_i$ release was not detected. Together our data demonstrate that (1) the effects of extracellular nucleotides on primary cultured keratinocytes may involve Ca^{2+} oscillations in a subset of cells and (2) elevation of extracellular Ca^{2+} facilitates agonist-induced Ca^{2+} entry in response to ATP-induced store depletion.

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Variations in the Genes Encoding the Peroxisome Proliferator-Activated Receptors α and γ in Psoriasis

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The three peroxisome proliferator-activated receptor (PPAR) subtypes α, β (or δ), and γ belong to the group of nuclear receptors that act as ligand-activated transcription factors. Recently, expression of PPARα and γ in keratinocytes has been demonstrated, and ligands of PPARα and γ were found to enhance epidermal maturation and protect against cutaneous inflammation. There is first evidence for a possible role of PPARs in psoriasis, as expression of PPARα and γ is decreased in lesional skin and treatment with PPARγ agonists improves psoriatic keratinocyte pathology *in vitro* and *in vivo*. We performed a case-control study to search for possible associations between variations in the genes encoding PPARα and γ and psoriasis. Seven variations in these genes were analyzed in 192 patients with chronic plaque-type psoriasis and 330 healthy controls by PCR-based methods. No association between any of the investigated *PPAR* variants and psoriasis was found. Our findings argue against a significant contribution of the investigated *PPAR* variations to the genetic basis of psoriasis.

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The Activity of the MAP-Kinases p38 and ERK1/2 are Increased in Lesional Psoriatic Skin

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Alterations in specific signal transduction pathways may explain the hyperproliferation and abnormal differentiation of the keratinocytes as well as the increased expression of inflammatory cytokines seen in psoriasis. Major signaling pathways used by eukaryotic cells to transduce extra cellular signals into cellular responses impinge on the mitogen-activated protein kinases (MAPKs).

The purpose of this study was to investigate the expression of the MAP-kinases p38, extra cellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK) in psoriatic skin. Using Western blotting we demonstrated increased levels of the activated (phosphorylated) form of p38 and ERK1/2 in lesional psoriatic skin compared to non-lesional psoriatic skin. No abnormality was found in the activation or expression of JNK1/2. *Ex vivo* kinase assays confirmed the increased activation of p38, and furthermore demonstrated increased kinase activity of the p38 isoforms p38α, p38β and p38δ in lesional compared to non-lesional psoriatic skin. p38γ was not detected in the psoriatic skin. Clearance of the psoriatic lesions, induced by climatotherapy at the Dead Sea for four weeks, lead to a normalization in the activity of both p38 and ERK1/2. Taken together, our results demonstrated that the activity of the MAP-kinases p38α, β and δ and ERK1/2 are increased in lesional psoriatic skin compared to non-lesional psoriatic skin, and that clearance of psoriasis normalizes the p38 and ERK1/2 activity. Thus, p38 and ERK1/2 might be potential targets in the treatment of psoriasis.

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The effects of Neuropeptides Galanin, Vasoactive Intestinal Polypeptide, Substance P and Calcitonin Gene-Related Peptide on the Productions of Nerve Growth Factor and Inflammatory Cytokines in HaCaT cells and Cultured Human Keratinocytes

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There is increasing evidence that the cutaneous neurosensory system can modulate physiological and inflammatory responses in the skin via the release of neuropeptides. Nerve growth factor (NGF) is an essential agent for the optimal function of keratinocytes and free cutaneous nerve endings. Since inflammatory cytokines play an important role in cutaneous inflammation, as does NGF in normal keratinocyte proliferation and wound healing, the effects of the neuropeptides galanin (GAL), vasoactive intestinal polypeptide (VIP), substance P (SP) and calcitonin gene-related peptide (CGRP) on NGF, IL-1α, IL-8 and TNF-α production were studied. Cultures of HaCaT cells and normal human keratinocytes were treated with 10^{-18} M GAL, VIP, SP or CGRP for 30 minutes. After washing, the cells were fed with fresh medium and their total RNA was isolated after 1, 3, 6, 12 and 24 h and subjected to reverse transcription. Quantitative real-time PCR reactions were performed with specific primer pairs and 18SrRNA as internal control. The NGF protein production was determined in the protein extracts of the cells by Western blotting and NGF secretion was measured by an ELISA technique. The results demonstrated that the neuropeptides have different effects on NGF production. In HaCaT cells, GAL and SP upregulated NGF mRNA expression, but CGRP and VIP caused no alterations. The positive effect of GAL on NGF protein production was verified by the Western blot assay, which showed more than a 2-fold protein amount after 24 h in GAL-treated cells. Interestingly, an increased NGF secretion could not be demonstrated in the cell culture supernatants. The positive effect of GAL on the keratinocyte NGF mRNA production was more marked, but the NGF secretion exhibited only a slight increase in the cell culture supernatants. Surprisingly, in contrast with previous results, none of SP, CGRP, GAL or VIP was able to influence the IL-1α, IL-8 and TNF-α mRNA levels significantly in HaCaT cells and cultured human keratinocytes.

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Immunohistochemical Analysis of EGFR and HER-2 in Patients with Metastatic Squamous Cell Carcinoma of the Skin

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Background. Metastatic squamous cell carcinoma of the skin often raise difficult therapeutic problems. EGFR and HER-2, which belong to the epidermal growth factor receptor family, are overexpressed in the most common tumors. Strategies have been developed to target HER-2 and EGFR with tyrosine kinase inhibitors. Few data are available about expression of EGFR and HER-2 in SCC of the skin. Overexpression of EGFR and of HER-2 proteins has been reported.

Objectives. The purpose of present study was to investigate the expression of EGFR and HER-2 in a series of metastatic SCC of the skin.

Methods. EGFR and HER-2 expression was studied by immunochemistry on 12 specimen of metastatic recurrence of SCC of the skin and on 2 primary lesions of these tumors.

Results. All metastatic SCCs of the skin had a strong membranous expression of EGFR. EGFR was also expressed in the two primary SCCs which were studied. HER-2 was weakly expressed in one third of the specimen only with a membrane expression in 2 cases.

Conclusions. In our study, EGFR was overexpressed in all samples of metastatic SCCs of the skin. Therefore, these metastatic tumors appear as suitable targets for treatment with tyrosine kinase inhibitors. Additional studies are warranted to establish whether or not HER-2 is expressed in SCC of the skin.

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Significance of Circulating T-Cell Clones in the Blood of Sézary Syndrome Patients

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Sézary syndrome (SS) is characterized by a circulating clonal CD4 + T cell population with skin homing properties. Recently, new phenotypic markers of Sézary cells have been described: CD158k/KIR3DL2, a MHC class I antigen receptor of the killer cell immunoglobulin-like receptor family, and SC5, a transmembrane receptor that is also over-expressed by normal activated T cells. CD158k has been shown to be a highly sensitive and specific marker of Sézary cells. Detection of clonal TCR gene rearrangement in the blood of SS patient is common. Whether circulating clonal T cells in SS are only malignant, or comprise normal activated lymphocytes is unknown. T-cell clonality is however widely used for the diagnosis and follow up of SS. The aim of this study was to determine the significance of circulating T cell clones in SS, using anti-TCRVB antibodies and the newly described phenotypic markers CD158k and SC5.

Thirteen SS patients were included in the study. Determination of T-cell clones was performed on peripheral blood mononuclear cells (PBMC) using a sensitive TCRVB complementarity-determining region 3 (CDR3) spectratyping and sequencing approach. One and two-color flow cytometry (FACS) analysis was used for TCR VB subfamily, CD158k and SC5 phenotyping on PBMC.

In all patients, one color FACS analysis showed that a major subset of CD4 + lymphocytes expressed CD158k and SC5. A total of 31 T-cell clones were identified by CDR3 spectratyping and sequencing. Unique (monoclonal) or multiple (monoclonal and oligoclonal) TCRVB subfamily CDR3 spectratype patterns were identified. Among these 31 T-cell clones, 19 were studied using the corresponding anti-TCRVB subfamily antibodies, and 13 were shown to be expressed. Two-color FACS analyses results were as follow : 1-) In 6 patients, coexpression of one clonal TCRVB with CD158k was present, thus identifying the malignant T-cell clone. In 3 of these patients however, CDR3 analyses identified, in addition to the malignant CD158k + T-cell clone, one or two minor T-cell clones. In all 6 patients, the malignant clonal population expressed SC5. 2-) In 5 patients, only normal T-cell clonal population were identified, characterized by a CD158k – SC5 + phenotype. In these patients, no CD158k + SC5 + malignant T-cell clone could be characterized. In one of these 5 patients, the T-cell clone remained detectable by CDR analyses even after clinical remission was obtained. 3-) In 2 patients, in whom 3 and 5 T-cell clones were detected by TCRVB CDR3 analysis respectively, TCRVB phenotyping failed to detect any of the clone.

This study shows that circulating clonal CD4 + T cells in SS can be either malignant or normal activated T-cell populations. TCRVB CDR3 analysis also identifies T-cell clones, either with monoclonal or oligoclonal CDR3 spectratype patterns, that are not phenotypically expressed. These findings may incite to limit the use of clonality and TCRVB phenotyping for the identification and follow-up of malignant T cells in SS, and rather perform CD158k and SC5 phenotyping.

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Fluctuations in Recent Thymic Emigrants in Peripheral Blood Measured Over Time, Correlates With Disease Activity in Atopic Dermatitis

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T-cell receptor excision circles (TREC) is an episomal DNA circle produced during T-cell receptor rearrangement and is used as a marker for recent thymic emigrants. To investigate peripheral T-lymphocyte turnover in patients with Atopic Dermatitis (AD), TREC in $CD4^+$ and $CD8^+$ T-cells was measured over time in a group of 8 patients.

Each patient was clinically scored with SCORAD and blood samples were obtained once a month over a period of six month. Peripheral blood mononuclear cells (PBMC) were isolated from the blood samples and subsequently $CD4^+$ and $CD8^+$ T-cells were isolated by positive immunoselection using Dyna-Beads. TREC levels were analyzed by real-time quantitative PCR. To normalize for input of DNA, the Cα constant region and $\beta_2$microglobulin were amplified in every sample tested. Correlations to disease activity parameters were done by Sperman Rank Order Correlation test.

The TREC content in $CD8^+$ T-cells declined with increasing AD disease activity judged by "Intensity" ($CC = -0.37$, $p = 0.018$) and "Extent" ($CC = -0.35$, $p = 0.023$), but not by SCORAD ($p = 0.06$). The same trend but no significant correlations were seen between the TREC content in $CD4^+$ T-cells and AD disease parameters. Furthermore, there is a good correlation between the variations in the TREC content in $CD4^+$ and $CD8^+$ T-cells over time ($CC = 0.9$, $p = 0.000$). As we have shown before the TREC content declines with increasing age.

These findings support our view that patients with AD can have an increased thymic output combined with an increased peripheral proliferation of their T-cells. The decline in $CD8^+$ TREC does not seem to be caused by mere dilution due to increase in leukocytes, as these were normal in AD. These new observations together with the previous findings support that there are severe changes in the T-cell homeostasis in the peripheral T lymphocyte system of AD patients.

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Evaluation of Treatment Efficacy in a Cohort of 281 Patients with Stevens-Johnson Syndrome (SJS) or Toxic Epidermal Necrolysis (TEN)

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None of the treatments proposed for SJS or TEN has been yet proven efficient. Having conducted a large case-control study of risk factors of these reactions, we also analyzed the impact of treatments given to these patients.

A total of 379 cases of SJS and TEN had been included, after the diagnosis and severity of the disease had been validated without information on risk factors, consecutive treatments and final issue. Systematic collection of information on the treatments given after hospitalization was organized for the 304 cases from Germany and France. Data were obtained in 281 cases (92.4%). In this cohort we evaluated the risk of dying in relation with the treatments administered, using a linear regression model including country and all variables that had an impact on prognosis, i.e. age and extent of illness. Among the 281 patients, 119 had been treated only with steroids, 75 had received high-dose intravenous immunoglobulins (IVIg) either alone (35 cases), or in addition to steroids (40 cas), 87 had supportive management only. Treatment modalities significantly differed between France (supportive 54%, steroids 19%) and Germany (supportive 12%, steroids 87%). The overall death rate in the group of patients analyzed was 22.1%, (62/281) i.e. similar to that of the 304 German and French patients (22%) and of the total EuroSCAR cases (21.6%). The risk of dying was significantly associated to age (OR = 3.3 [1.5–7.5] for 40–70 years et OR = 8.9 [3.7–21] above 70), to extent of disease (OR = 2.3 [1.0–5.1] for SJS-TEN overlap and OR = 5.6 [2.4–13] for TEN). Mortality stratified by treatment

	Mortality (%)	OR Univariate	OR multivariate
Supportive only	22/87 (25%)	1	1
IVIG alone	12/35 (34%)	1.5 (0.7–3.6)	1.6 (0.6–4.3)
IVIG + steroids	7/40 (18%)	0.6 (0.2–1.6)	0.5 (0.1–1.5)
Steroids alone	21/119 (18%)	0.6 (0.3–1.2)	0.4 (0.2–1.1)

This is the largest cohort ever analyzed. With the limitations inherent to observational studies this is the best available evidence. The results show that IVIG confer no benefit towards supportive care and that steroids may deserve formal therapeutic trials.

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***In Vitro* IL-5 Release in Patients with Cutaneous Adverse Drug Reactions – A Comparison with *In Vitro* IFNγ Release**

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Drug specific T-cells may orchestrate the inflammatory skin reactions in cutaneous adverse drug reactions (CADRs) through release and induction of different cytokines. The diagnostic value of IFNγ (a Th1-type cytokine) release test has been reported in Th1 and Th2-type reactions. IL-5 (a Th2-type cytokine) may also be of diagnostic value in CADRs.

This study was conducted in order to determine *in vitro* secretion of IL-5 by patients with different CADRs after *in vitro* challenge with the suspected drugs and furthermore to compare the *in vitro* IL-5 release test with the *in vitro* IFNγ release test.

Sixteen patients who were diagnosed as having CADRs after taking 17 different drugs (1–5 drugs per patient), were included in this study. The CADRs were divided into Th1-type reactions: maculopapular rash (5 patients), Th2-type reactions: urticaria (5 patients) and immune complex reactions: vasculitis (6 patients). Sixteen patients who took 15 different drugs (1–5 drugs per patient), without developing an adverse reaction served as controls. Peripheral blood lymphocytes were incubated with the suspected drugs. The concentration of IFNγ and IL-5 in the supernatants was determined by ELISA commercial kits. The increase of cytokine concentration after *in vitro* challenge with the suspected drugs was calculated for patients and controls. A value higher than the average cytokine secretion by the controls plus two standard deviations was defined as a positive cytokine release test. 33 cytokine release tests (IL-5 and IFNγ) were performed in the group of patients as compared to 24 tests in the controls. IL-5 test results were compared to IFNγ test results of the same patients. The study was conducted in a double blind manner.

IL-5 secretion was significantly higher ($p < 0.0001$) in patients compared to controls. Positive IL-5 responses were obtained for a variety of drugs and CADRs: sensitivity 62.5%; specificity 93.7%. The distribution of positive IL-5 responses according to the type of CADR was: urticaria 80% (4/5), maculopapular rash 40% (2/5), vasculitis 66.6% (4/6). Positive IFNγ responses were obtained for a variety of drugs and CADRs: sensitivity 81.25%; specificity: 87.5%. The distribution of positive IFNγ according to the type of CADR was: urticaria 80% (4/5), maculopapular rash 60% (3/5), vasculitis 100% (6/6). Agreement between IL-5 test and IFNγ test results was 42.5% (kappa = 0.11; poor agreement).

The results of this study indicate that IL-5 is released *in vitro* after challenge with a variety of drugs in Th1-type and Th2-type CADRs. IL-5 test is less sensitive than IFNγ test and is more sensitive in patients with Th2-type CADRs compared to patients with Th1-type CADRs.

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Chemosensitization of Malignant Melanoma by Bcl-2 siRNA

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Malignant melanoma is a highly chemoresistant type of tumor and with currently available treatment options the prognosis is poor: dacarbazine (DTIC), as the only FDA approved chemotherapeutic agent for melanoma, results in low response rates and the duration of responses is short. Even with innovative combinatorial treatment regimes, no fundamental breakthrough has been reached. More than 90% of all melanomas express the anti-apoptotic protein Bcl-2 shown to contribute to a chemoresistant phenotype in melanoma and a variety of other malignancies. We have previously demonstrated, that inhibition of Bcl-2 by antisense oligonucleotides provides a promising strategy to sensitize malignant melanoma to several apoptosis inducing treatment modalities. Over the last couple of years, small interfering RNA (siRNA) emerged as a novel and even more powerful technique for targeted downregulation of target mRNA. We therefore evaluated synthetic siRNA molecules targeting Bcl-2 as strategy to silence Bcl-2 expression in malignant melanoma cells. In a screening approach we identified two siRNA with high potency for specific downregulation of Bcl-2 expression in melanoma cells. These siRNA resulted in an up to 15 fold reduction of bcl-2 mRNA levels and only still barely detectable Bcl-2 protein expression at low nanomolar concentrations. In cell growth experiments, silencing of Bcl-2 in melanoma cells by both siRNAs resulted in only moderate inhibition of cell growth and apoptotic cell decay, respectively. In contrast, if siRNA targeting Bcl-2 were combined with low doses of the apoptosis inducing chemotherapeutic cisplatin, a massive increase of apoptotic cell death compared to controls was observed. Notably, the combination of the Bcl-2 targeting siRNA with low dose cisplatin resulted in a nearly complete suppression of cell growth whereas cell growth of cisplatin only treated cells was only modestly affected (97% vs. 24%; $p < 0.001$).

Our findings underline a key role for Bcl-2 in conferring chemoresistance to melanoma and highlight Bcl-2 siRNA strategies as novel and highly effective tools with the potential for future targeted therapy of malignant melanoma.

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Somatostatin Receptor Scintigraphy with ^{111} In-DOTA–Lanreotide and ^{111}In–DOTA –Tyr^3 -Octreotide in Patients with Melanoma Stage IV: *In Vitro* and *In Vivo* Results

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Background. The overexpression of somatostatin receptors (SST-R) on various tumor cells provides the molecular basis for the successful use of radiolabelled SST analogues in clinical oncology. The aim of this study was to evaluate the tumor binding of ^{111}In-DOTA-Lanreotide (DOTA-LAN) and ^{111}In-DOTA-Tyr^3-Octreotide (DOTA-OCT) in patients with melanoma stage IV. In addition, we have performed *in vitro* evaluations of potential antiproliferative effects of SST analogues along with assessment of the functionality of SST-R in malignant melanoma.
Patients and Methods. A total of 22 patients with advanced metastatic melanoma underwent scintigraphy with a radiolabelled SST-analogue. In addition, *in vitro* binding, growth inhibition and influence of the SST analogues LAN and OCT on cell cycle distribution were performed in four melanoma cell lines (SK-MEL28, 518A2, JUSO, 607B).
Results. Thirty-eight out of 57 lesions were positively imaged with ^{111}In-DOTA-LAN (67%), while the remaining 19 (33%) were negative. With ^{111}In-DOTA-OCT scintigraphy, 8 (44%) of the 18 documented lesions were imaged, whereas the remaining 10 (55%) had no uptake. *In vitro*, cell lines ($n = 4$) showed no inhibition of growth in presence of LAN or OCT, and no influence on cell cycle distribution was found despite detection of mRNA for SSTR1, 2 and 4 using PCR. Fluorescence-labelled LAN was not internalised after binding to the cell surface of the melanoma cell lines.
Conclusions. ^{111}In-DOTA-LAN and ^{111}In-DOTA-OCT were able to visualize melanoma metastases in a high proportion of patients. By contrast, *in vitro* experiments did not reveal functional surface SST-R in the four cell lines investigated, and SST-analogues did not influence growth or cell cycle distribution.

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Imiquimod Treatment Induces Opioid Growth Factor Receptor Expression – a New Way to Defeat the Tumor?

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Imiquimod is a local immune response modifier that has demonstrated efficacy in clearing superficial basal cell carcinoma (sBCC). Via interaction with Toll-like receptor 7 on immune cells, imiquimod induces local production of cytokines, such as interferon (IFN)-α. To more closely define and elucidate mechanisms leading to BCC clearance *in vivo*, we examined gene expression profiles of skin sBCC before and after treatment with 5% imiquimod cream (Aldara™, 3M Pharmaceuticals, St. Paul, MN) by using Affymetrix™ HG-U95A high-density oligonucleotide arrays that contain approx. 12'000 genes. Imiquimod predominantly induces genes involved in different aspects of immune response. In addition, array data indicated that imiquimod treatment induces expression of opioid growth factor receptor (OGFr), a molecule with anti-proliferative properties recently reported to be a target for anti-tumor antibody responses. By using quantitative PCR (LightCycler™) we confirm OGFr up-regulation in Aldara-treated sBCC skin samples. Immunohistochemistry revealed *in vivo* up-regulation of OGFr protein on tumor and on infiltrating cells after Aldara treatment. By using BCC cell lines treated with IFN-α or imiquimod, we show that up-regulation of OGFr mRNA as well as OGFr protein is IFN-α- rather then directly imiquimod-mediated. By using tissue microarray containing 52 BCCs, we demonstrate OGFr expression in almost half of the cases. Expression of OGFr correlated with a longer recurrence-free period in BCC that recurred after radiotherapy. Through IFN-α–mediated up-regulation of OGFr, imiquimod might not be only suppressing tumor growth but it might as well increase tumor immunogenicity.

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Monocyte Derived Dendritic Cells Mature in Response to the TLR7 Agonist Loxoribine

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Introduction: Dendritic cells (DCs) are potent antigen-presenting cells recognizing micro-organisms, secreting pro-inflammatory cytokines and stimulating primary T cell immune responses. Therefore DCs are potential candidates in the immunotherapeutic approaches for cancer. Current vaccination trials have tested myeloid DCs in advanced tumour patients. Optimal DC vaccination parameters in humans remain to be established. One of the open questions is the optimal maturation of DC preparations. A group of transmembrane proteins called Toll-like receptors (TLRs) can relay critical signals for regulating DC functions such as maturation status. Monocyte derived myeloid DCs express a whole set of TLRs but there is controversy concerning their TLR7 expression. Substituted low-molecular-weight nucleosides activate immune cells and lead to cytokine secretion. Loxoribine, a potent guanosine analog, which signals through TLR7, shows antiviral and anti-tumor activity in murine animal models and has entered phase I clinical trials.
Aims Of The Study. To define the TLR7 expression of myeloid DCs and to define the maturation status of myeloid DC preparations after loxoribine incubation.
Materials And Methods. Monocyte derived DC cultures were set up using IL-4 and GM-CSF. At day 7 immature DC cultures were treated with a standard maturation cocktail (IL-4, GM-CSF, TNF-alpha, IL-1beta, IL-6) and/or different loxoribine concentrations. A maturation cocktail without IL-6 was also tested with or without different loxoribine concentrations. FACS analysis of surface co-stimulatory molecules of DCs was performed on day 10. In other sets of experiments RNA was isolated from monocytes and from DC cultures at different time points. After reverse transcription real time quantitative PCR was performed using specific human TLR7 primers.
Results And Discussion. Loxoribine treatment of DCs resulted in increased cell surface expression of CD80, CD83 and CD86. DCs stimulated with TNF-alpha, IL-1beta plus loxoribine had a similar phenotypic characteristics as of DCs stimulated with the standard maturation cocktail and their CD86 expression was even higher. Our real time PCR results showed that purified monocytes and myeloid DCs derived from them express TLR7 on the mRNA level and that myeloid DCs upregulate their TLR7 mRNA expression after loxoribine treatment. These results suggest that loxoribine is a likely candidate for use as an adjuvant for DC-based cancer immunotherapy.

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An Investigation of PIIINP as a Marker of Hepatic Fibrosis in Psoriasis: Does the Arthritis Affect PIIINP Levels?

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Background: A serum marker of collagen turnover, Procollagen III

N terminal peptide (PIIINP) is used principally by dermatologists as a marker of hepatic fibrosis to reduce the need to perform liver biopsies. Serial negative values have been found to be of value in excluding hepatic fibrosis in psoriatic skin disease.[1] Absolute PIIINP level is used to make dosage decisions on psoriatic patients in the Dermatology department which impacts on arthritis disease activity. PIIINP is found in the joint fluid in active arthritis and has been reported as elevated in active psoriatic arthritis, reducing its utility in this group. For patients with known hepatic histology we investigated its relation to joint disease activity to investigate the hypothesis that any elevation in PIIINP may be due to joint disease activity. Methods: We recruited 57 psoriasis patients under joint review by dermatologists and rheumatologists, who were scheduled to have a liver biopsy performed to monitor long-term methotrexate from Oct 2002 to March 2004. All patients were clinically assessed for joint disease activity by tender joint count, swollen joint count and entheseal index. This was compared to the serum PIIINP level on the day of the assessment, prior to liver biopsy. CRP was also measured. Results: 48 patients with treated psoriatic arthritis and 9 with psoriasis alone (6 male 3 female). Total 57. 16 (33%) of 48 arthritis patients had one or more of the following: 5 or more swollen joints, 5 or more painful joints or an entheseal index greater than 5 to suggest disease activity. There was no correlation between PIIINP value swollen joint count ($P = 0.99$) tender joint count ($P = 0.74$) entheseal index ($P = 0.56$), or CRP ($P = 0.90$). There was a marginally significant difference between the PIIINP values of patients scoring 1, 2 and 3 on the Roenigk scale (Kruskal-Wallis test $P = 0.06$). There was a significant trend towards higher PIIINP being associated with higher Roenigk score (Spearman correlation $P = 0.02$ Conclusions: This group of patients are representative of those under follow-up by rheumatologists across the UK with psoriatic arthritis treated with long term methotrexate. In this study the PIIINP level was often elevated but this was not explained by either liver fibrosis or by the presence or severity of psoriatic arthritis. Elevated levels occurred in the absence of either arthritis or hepatic fibrosis. Regular PIIINP monitoring may still be of use in psoriatic arthritis.

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The Incidence of Methotrexate Induced Hepatotoxicity in Psoriatic Arthritis

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Background: The aims of this study were to investigate the incidence of hepatic fibrosis in psoriatic skin and joint disease treated with long-term methotrexate therapy. In addition we aimed to assess known risk factors for MTX induced hepatotoxicity as well as those for steatohepatitis (NASH), a new clinical entity seen in obesity which may confound histological appearances. Methods During a 2 year period in two hospitals patients attending both rheumatology and dermatology outpatients ($n = 57$) on a stable dose of long-term methotrexate had liver biopsies performed in accordance with British society of Dermatologist guidelines. Clinical examination including BMI, waist and hip measurement, urine dipstick to exclude diabetes. Laboratory analysis including LFT's, clotting studies, CRP, FBC and a PIIINP (procollagen III-N terminal peptide) Results 57 patients with psoriatic disease had liver biopsies. Males = 32 Females = 25. Five males and 4 females had skin disease only. Median age was 55 (range 30–78). Mean cumulative dose (4334mg). Mean weekly dose 14.8mg. Mean BMI 30.7. 14 patients had BMI greater than 30. Four patients had type 2 diabetes, 4 had renal impairment.

Results of liver biopsy showed that 13 patients of 5 (22.8%) had mild early fibrosis while 1 of those had bridging septal fibrosis. There were no cases of clinical liver disease or cirrhosis found. Comparing those with presence of hepatic fibrosis and those without hepatic fibrosis there was no significant difference with regard to age, sex, weekly dose or duration of therapy. Patients with hepatic fibrosis had significantly lower cumulative dose and also consumed fewer units per week of alcohol than those without. Those patients with a greater number of recognised risk factors for hepatic fibrosis were more likely to have fibrosis (renal impairment, DM, Obesity). Conclusions This study shows that the incidence of subclinical mild hepatic fibrosis on methotrexate therapy is as common as previously shown. There was no evidence that this was linked to methotrexate therapy alone. Neither is it suggestive of alcohol as a major aetiological factor. The aetiology of fibrosis in these patients may be multifactorial including non-alcoholic steatohepatitis.

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Cumulative Skin Irritation Potential of XMP.629, a New Antimicrobial Peptide for the Treatment of Acne Vulgaris

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Antibiotics are not as useful as they once were for acne treatment as *Propionibacterium acnes (P. Acnes)* has developed antibiotic resistance. A significant opportunity exists for innovative, effective, and safe topical agents that have no side effects and avoid antibiotic resistance. XMP.629 is a nine amino acid peptide derived from bactericidal/permeability-increasing protein (BPI), a human host defense protein localized within the azurophilic granules of neutrophils. It has potent antibacterial activity against *P. acnes* and neither clindamycin nor erythromycin resistance influences the sensitivity of *P. acnes* to XMP.629.

This phase I, single center, evaluator-blind study assessed the cumulative skin irritation potential of XMP.629 gel (0.1%), vehicle and positive control (0.3% sodium lauryl sulfate) following repeated topical applications to 35 healthy subjects. The test articles were applied with occlusive patches to the subjects' backs over 3 weeks. Patches were removed after 48 hours (72 hours when applied on Fridays) and evaluations of skin reactions on the test site were made on a six-point scale. There were a total of 9 readings during the 3-week period.

Mean irritation scores were tabulated by test article and evaluation day and summed across days for each test article. The cumulative irritation score for each test article is the total irritation score divided by the highest theoretical score. No sites were discontinued due to the severity of the irritation and no subjects were discontinued for any reason. Subjects with barely perceptible erythema (score of 0.5) were counted as having slight erythema (score of 1). The mean cumulative irritation score was tested pair-wise for product differences using Fisher's protected least significant differences in the context of the two-way analysis of variance (ANOVA).

The results demonstrated that the mean cumulative irritation score for XMP.629 gel (0.1%) was 0.08. This score was comparable to the mean cumulative irritation score for vehicle gel (0.07) and markedly lower than the mean cumulative irritation score for the positive control 0.3% sodium lauryl sulfate, 0.25 ($p < 0.001$).

We conclude that XMP.629 acetate gel (0.1%) is well tolerated and should continue to be evaluated for the treatment of acne vulgaris.

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Pruritic Skin Conditions During Pregnancy: Serum Bile Acid Levels are a Highly Reliable Parameter to Diagnose Intrahepatic Cholestasis of Pregnancy (ICP)

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Pruritic skin changes are a common condition during pregnancy. Most of them are benign and self-limiting with no impact on fetal outcome. ICP, however, may cause severe fetal distress and carries a risk for stillbirth and preterm delivery. Early diagnosis and treatment are therefore essential. Its clinical presentation with pruritus and secondary skin lesions (excoriations, prurigo) in late pregnancy is unspecific and may be confused with specific dermatoses of pregnancy and atopy-related skin changes.

To evaluate the significance of elevated serum bile acid (SBA) levels in the diagnosis of ICP.

SBA levels (assessed by RIA; normal range 0–6μmol/l) of 75 female patients (mean age 28.7y, range 15–44y) seen from 1999–2004 for pruritic skin changes during pregnancy at a specialized dermatologic pregnancy clinic in a university-based hospital were retrospectively analysed with special interest to diagnosis and clinical presentation. Dermatologic diagnoses were as follows: specific dermatoses of pregnancy (14pts.), ICP (11), atopy-related skin changes (46) and others (4): drug reaction (1), pityrasis rosea (1), psoriasis (2).

Elevated SBA levels were found exclusively in all patients with ICP (mean 37.4μmol/l, range 7.3–138μmol/l) compared to the other diseases (mean 2.3μmol/l, range 0.4–4.5μmol/l) [$\chi^2 = 75$, $p = 0.000$].

As the clinical presentation of ICP is overlapping with other pruritic skin conditions during pregnancy, the diagnosis should be based on elevated SBA levels, which have proven to be a highly reliable parameter.

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Sentinel Lymph Node Biopsy in Melanoma Patients with Clinically Negative Regional Lymph Nodes – Analysis and Importance of Sentinel Node Status

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Sentinel lymph node biopsy has become a widely accepted standard procedure in staging and treatment of patients with cutaneous melanoma and absence of lymph node metastases, despite there is no final proof that sentinel node biopsy may influence overall survival of these patients. This study investigated the accuracy of this procedure and the clinical outcome of patients after a mean follow up of 22 months.

Between 1998 and 2003, sentinel node biopsy was performed in 309 consecutive patients. Patients with one or more positive sentinel nodes, identified by concurrent histopathology and immunohistochemistry, were subjected to completion elective lymph node dissection. Survival analyses were performed using the Kaplan-Meier approach. A Cox-proportional hazard analysis was used for univariate and multivariate analysis to explore the effect of variables on survival.

One or more sentinel nodes were identified in 299 of 309 patients (success rate: 96,8%). Of these, 69 (23%) had a positive sentinel node. The false-negative rate was 10%. Recurrence of disease to the regional lymph node basin (3.5%) and to the locoregional skin (2,6%) was rare in SLN- negative patients in contrast to SLN- positive patients (7.2%, and 17.4%, respectively). Three-year disease-specific survival was 93% and 83% for SLN-negative and SLN-positive patients, respectively. Upon multivariate analysis, sentinel node status ($p < 0.001$), Breslow thickness ($p < 0.02$), and ulceration ($p < 0.026$) were all found to be independent prognostic factors in respect to disease-free survival, whereas Breslow thickness proofed to be the only significant factor in respect to disease-specific survival.

SNB proofed to be a reliable procedure for accurate staging of nodal status in melanoma patients without evidence of nodal disease. Our data indicate that the status of the SLN node is predictive for disease-free survival but not for disease- specific survival, upon multivariate analysis. Final results from ongoing trials are needed to clarify the role of lymphatic mapping and sentinel node biopsy on overall survival for patients with malignant melanoma.

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Electrically Evoked Itch Indicates the Existence of Novel C-Nerves for Itch

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Histamine is the best known pruritogen. Previous studies investigating itch mechanism in human beings were based mainly on histamine and histamine-releasing substances. Recently, a specific neuronal pathways for histamine-induced itch have been identified. However, antihistamines are of limited value in many pruritic diseases like atopic dermatitis, suggesting a minor role of histamine as a clinical pruritogen. The purpose of this study was to investigate mechanism of non-histaminergic itch evoked by a newly developed electrical stimulation on human skin.

Electrical stimulation with constant current (2 ms, 50 Hz) was applied via a 0.1 × 7 mm electrode to non-lesional wrist skin in ten patients with atopic dermatitis and ten healthy volunteers. As a control, histamine iontophoresis was applied through a 0.1 to 0.2 × 7 mm slit to the wrist skin of the same ten healthy volunteers. The axon-reflex flare was measured by a laser Doppler imager. The area of alloknesis (itch evoked by non-itchy mechanical stimuli) was planimetrically measured. Electrical stimulation with different pulse duration (80 micros to 8 ms) and frequency (2 Hz to 200 Hz) was also applied and the intensity of evoked sensation was compared among them.

Itch was the main sensation evoked by electrical stimulation and always perceived with a time lag of approximately one second compared to the stimulation. There was no significant difference observed in evoked reactions between healthy volunteers and patients. Itch intensity was controlled by varying current intensity. Electrically evoked itch was not accompanied by axon-reflex flare, while histamine iontophoresis provoked a marked flare reaction. The area of alloknesis induced by electrical stimulation was larger than by histamine iontophoresis. At a given current intensity, 2 ms or longer pulse duration was found to be necessary to evoke itch sensation. The intensity of itch sensation was proportional to the frequency of stimulation.

Itch can be evoked by our newly developed electrical stimulation without any involvement of peripheral chemical mediators like histamine. The time lag between stimulation and itch sensation and the necessity of longer pulse duration for evoking itch suggest that C-fibers are responsible for electrically evoked itch. However, these fibers are not identical to the previously described histamine sensitive fibers, because their low electrical threshold and the absence of an axon reflex erythems. It can be concluded that this method is useful to investigate non-histaminergic itch mechanism.

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Cutaneous Field Stimulation; Effects on Skin and Spinal Ganglia in Rat

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Cutaneous field stimulation is used to treat localized itch. The aim of the present study was to determine whether such treatment induces morphological and neurochemical changes in the dorsal root ganglia in 30 rats. Electrical stimulation using 0.13 mA or 0.53 mA, 30 min a day for 10 days. Punch biopsies from the thoracal skin and the corresponding dorsal root ganglia were collected post mortem. In both experimental groups, cutaneous electrical stimulation induced a proliferation of sensory nerve fibers in the skin. The mean number of PGP 9.5-IR nerve fibers was 312±52 and 291±67, respectively vs. 209±47 in the controls, the number of CGRP-IR nerve fibers being 66±19 and 66±16, respectively vs 40±14 in the controls. Only stimulation by 0.53 mA induced an upregulation of sensory neuron markers in the dorsal root ganglia, the lower stimulation intensity having no visible effect on ganglia. The mean ratio of pos/neg PGP-IR cells was 41±8% vs 35±6 in controls, the mean ratio of pos/neg CGRP-IR cells was 37±6% vs. 33±5, and the mean ratio of pos/neg VR1-IR cells was 46±6% vs. 42±5. We conclude that serial cutaneous electrical stimulation by a moderate current in rat does not induce morphological and neurochemical changes in the dorsal root ganglia.

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Unique Distribution of Cytochrome P450 1B1 and 2A6 in Extramammary Paget's Disease

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Cytochrome P450 1B1 (CYP1B1) is a recently cloned dioxin-inducible form of CYPs and now known to be expressed as enzyme protein not only in malignant tumor cells but also in several normal cells including neurons, astrocytes and glandular epithelial cells of the breast. The enzyme is responsible for metabolic activation of procarcinogens. Cytochrome P450 2A6 (CYP2A6) activates several anti-cancer drugs such as Tegafur metabolically. Therefore the investigation on the expression profile of these enzymes in skin cancers may provide useful clinical information and realize a rational anti-cancer therapy.

Thirty-six cases of extramammary Paget's disease (EMPD) were studied by immunohisitochemical technique using anti-human CYP1B1-specific peptide rabbit serum (Gentest, MA, USA) and anti-human CYP2A6 mice monoclonal antibody (Gentest, MA), respectively. Anti-CYP1B1 antiserum consisitently reacted with smooth muscle cells and eccrine sweat gland cells in the skin so that they could be used as positive internal control. Tumor cells of EMPD were well stained with good contrast towards surrounding epidermal keratinocytes which showed less or no immunoreactivity (24 cases/total 36 cases). Eccrine gland secretory cells were positively reacted by anti-CYP2A6 antibody. In case of EMPD, tumor cells in 21 cases were positive for anti-CYP2A6 antibody. These data will be helpful for the development of new effective method for diagnosis and drug therapy in EMPD.

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An Examination of Pigmentary and Non Pigmentary Response to UV Radiation in Humans

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The roles of pigmentation and hyperplasia in the photoprotection of skin are not completely understood; although, it is known that both play a vital role in the protection of skin cells from UV insult. An attempt has been made here to increase the understanding of these processes using UV absorption spectroscopy. Suction blisters were removed from the lower back of volunteers, and placed into a saline filled cavity within a quartz cuvette. Equal UV doses were applied to 21 healthy volunteers including 4 asians and 17 caucasians, of whom 3 had red hair. The absorption spectrum (250nm–400nm) of the irradiated area was compared with that of an adjacent control area in each case.

A mathematical model allowed the calculation of the thickness ratios and the melanin ratios of any two pieces of skin. Different responses were observed for different skin types. Caucasian skins showed no increase in melanin, but a significant increase in thickness when normalised to a given skin sample: 0.99 (SEM 0.07) to 1.27 (SEM 0.08) ($p = 0.004$), while asian skins showed no thickness increase yet a melanin increase, although this was not significant: 2.75 (SEM 0.57) to 2.98 (SEM 0.46) ($p > 0.01$).

Probability of DNA damage, P, was calculated (Setlow 3363–66), and the asian skins had the lowest P overall: 1.16×10^{-7} (SEM 0.08×10^{-7}); however, the red haired caucasian skins showed the greatest decrease in P on tanning: 2.49×10^{-7} (SEM 0.17×10^{-7}) to 1.47×10^{-7} (SEM 0.18×10^{-7}) ($p = 0.002$), indicating greatest levels of photoprotection. When split into the individual components of melanin, scatter and thickness, the results mirrored those obtained using the ratio calculations.

Correlation between the two models was excellent, although poorer in general for the darker skins; however, since sun damage and related skin cancers are found almost exclusively amongst the caucasian populations, the model provides an accurate measure of the effect of UV exposure on epidermal hyperplasia and pigmentary response for those at most risk from sun damage. Both pigmentation and hyperplasia can play a role in photoprotection; however, hyperplasia resulted in red haired caucasians exhibiting the highest levels of photoprotection on irradiation.

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Possible Mechanisms of Action of Iloprost in the Treatment of Patients with Systemic Sclerosis – Influence on the Expression of Adhesion Molecules and T-cell Proliferation

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Iloprost is a stable prostacyclin analogue commonly employed in the treatment of peripheral vascular disease: It has also been used successfully in the treatment of severe Raynaud's phenomenon (RP) associated with systemic sclerosis (SSc). Several mechanisms of action of the drug other than vasodilation and antiplatelet effects have been demonstrated that may be involved in its clinical efficacy.

The aim of the present study was to investigate the long-term influence of Iloprost infusions on disease activity related factors like expression of soluble adhesion molecules and T-cell reactivity.

Serum blood samples were collected before therapy and at time points 0, 1, 2, 3 and 6 months after therapy and examined for Endothelin-1 (ET-1), Vascular Endothelial Growth Factor (VEGF) and soluble adhesion molecules sICAM-1, sVCAM-2 and sE-Selectin using specific enzyme-linked immunoassays.

Human PBMC of patients with systemic sclerosis were incubated with three stimuli to cover different ways of T-cell activation: non-specific (PHA), receptor mediated (IL-2) and antigen specific receptor mediated (tetanus toxoid (TT)). Activation of PBMC was evaluated by measuring the proliferation in a BrdU-ELISA as well as cytokine production by either an IFNγ or IL-4 ELISA.

All patients showed a clinical reduction in severity and number of Raynaud attacks. Mean serum levels of sE-Selectin, sVCAM-1, sICAM-1 and ET-1 were reduced after therapy with Iloprost. This effect persisted at least for two months (ICAM-1). Serum levels of sE-Selectin, VCAM-1 and ET-1 were still reduced at the end of the study period after 6 months. Serum concentration of VEGF was found to be elevated after infusion with Iloprost reaching the initial level 2 months after infusion.

The effect of Iloprost on the proliferation of PBMC showed great interindividual differences. However, stimulated with 150 ng/ml Iloprost, 10 of 11 patients showed a decreased proliferation. Similar effects – although not as pronounced – could be seen when cells were stimulated with TT and IL-2. Comparison of the mean values of PBMC proliferation obtained from the same patients after treatment with Iloprost revealed reduced proliferation when stimulated by PHA and no changes when stimulated by TT and PHA. No effects of Iloprost on the cytokine production could be seen at any time-point.

These results give further evidence of the previously described clinical benefit of Iloprost infusions above all on RP in patients with SSc. Our study suggests that these effects might be related to changes in serum levels of disease activity related markers and T cell proliferation and can be followed for a prolonged interval after infusion therapy.

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Successful Treatment of Hereditary Angioedema with a Bradykinin Receptor Antagonist

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Bradykinin seems to play an important role in the pathogenesis of hereditary angioedema due to C1 inhibitor deficiency: Bradykinin plasma levels are increased during acute attacks and in a recently developed C1 inhibitor knock-out mouse model, it has been shown that Icatibant, a bradykinin B2 receptor antagonist, reduced fluid extravasation and edema formation. We treated 11 acute edema attacks, moderate or severe, in 10 patients with hereditary angioedema with Icatibant (Jerini AG, Berlin, Germany), a dekapeptide which is highly specific for the bradykinin B2 receptor. Treatment was started less than 10 hours after the beginning of the edema episodes. The patients received Icatibant (8 patients 0.4 mg/kg body weight; 3 patients 0.4 mg/kg body weight) intravenously as an infusion. The course of the treated edema episodes was evaluated using questionnaires, visual analogue scales and photodocumentation. We compared the usual course and duration of the treated skin and abdominal attacks with that of edema attacks that had occurred previously in the same patients at the same location as the current attack (10 to 600 attacks per patient). Treatment with Icatibant considerably shortened the time between beginning of the attack and of the resolution of symptoms (untreated attacks: mean: 41.3 hrs, treated attacks: mean: 10.7 hrs). The duration of the treated attacks was also shorter than that of previous, untreated attacks (untreated attacks: mean: 73.5 hrs, treated attacks: mean: 24 hours). No drug-related adverse effects were reported. The drug may be of considerable therapeutic value for patients with hereditary angioedema.

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Certain Severity-Related Parameters in Atopic Dermatitis: Possible Candida Role and its Theraputic Response

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Objective: To evaluate certain severity-related markers of atopic dermatitis (AD) in relation to Severity Scoring (SCORAD) and to clarify the relationship between *Candida albicans* colonization and severity of AD with the effect of antifungal treatment on previous markers.
Design. A prospective study included 82 AD patients and 20 healthy controls. Patients were grouped into two groups according to the severity of the disease. SCORAD index was detected for each. Blood samples were taken for detection of total eosinophilic count, total IgE , *Candida albicans* specific IgE, intercellular adhesion molecule-1 (ICAM-1) and eosinophil cationic protein (ECP). Isolation and identification of *Candida albicans* was done, then an oral antifungal drug (Fluconazole) was given for 4 weeks with monitoring changes in these parameters.
Results. There was significant increase in ECP, ICAM-1, total eosinophilic count, total IgE levels in AD patients as compared with control especially the severe group ($P < .001$). Serum levels of ECP, ICAM-1 represented high significant positive correlation with SCORAD. Significant high incidence of *C. albicans* isolates were detected in both AD groups (Severe group 40%, mild-moderate group 36.7%) with significant increase in *C. albicans* specific IgE that could be correlated with SCORAD ($P < .001$). With Fluconazole, there was mild to moderate significant improvement of SCORAD, decrease of ECP & ICAM-1 with high sgnificant decrease in *C. albicans* specific IgE.
Conclusions. Eosinophils are important effector cells in AD. The serum levels of ECP and ICAM-1 have been proven to be high significant predictors of disease severity. Isolation of *Candida albicans* and high level of its specific IgE especially in severe AD group that could be correlated with SCORAD confirms a possible role of antifungal treatment of AD with positive *C. albicans* isolation.

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What Factors may Contribute to the High Degree of Variation in Erythemal Response to Ultraviolet Radiation (UVR) at Different Body Sites?

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We have previously reported a fivefold variation in erythemal response to UVR at different body sites within one person. We have explored two possible reasons for this; firstly, that body site variation in constitutive pigmentation partly determines some of this variability; secondly, that resting dermal microvasculature flow variation may relate to the UVR induced vascular response at a particular site.

To explore variation in pigmentation and its relation to erythema we irradiated 4 volunteers with 10 doses (38–300mJ/cm^2) of broadband UVB (PLS 9w/12) at 12 paired body sites (240 potential data points per person) and measured the melanin index (MI) on closely adjacent unirradiated skin. Erythema was measured at 24hrs using reflectance. To see if basal pigmentation at one site could predict erythema at other body sites we irradiated 15 volunteers at 3 sites with 5 doses of UVB (119–300mJ/cm^2) and recorded the colour of sun protected buttock skin with tristimulus colorimetry (L*a*b*) and MI. Erythema was measured at 24hrs using laser Doppler flowmetry. To see if variation could be explained by differences in regional basal blood flux differences we recorded Doppler flowmetry at 9 body sites in 9 volunteers.

When the MI was recorded adjacent to the irradiated body site, simple regression of erythema on MI was highly significant for each individual (all $p < 0.001$, R^2 of 0.75–0.83) for the highest dose (300mJ/cm^2). Yet L*a*b* and MI measurements of basal (buttock) skin colour showed a poor correlation with erythema at other sites (Spearman's rank correlation < 0.3) suggesting that basal skin colour cannot usefully predict erythema elsewhere. Baseline blood flux showed a mean 3-fold variation (range 16–52) but the ranking of sites differed from that of irradiated body sites. Therefore, baseline variation in flux cannot account for the degree of variation in UVR induced erythema.

We conclude that body site variation in pigmentation, as measured by the MI, is a major contributory factor to the variation in erythemal response to UVR within a person at different body sites.

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Efalizumab for Patients with Moderate to Severe Chronic Plaque Psoriasis: Results of the International Randomised, Controlled Phase III Clinical Experience Acquired with Raptiva (Clear) Trial

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Psoriasis is a chronic immune-mediated skin condition that requires treatment over decades. No curative agents are available, and long-term administration of the most effective systemic agents or phototherapy is generally limited due to toxicity. Efalizumab is a recombinant humanised monoclonal IgG_1 antibody that inhibits key T-cell processes that contribute to the pathogenesis of plaque psoriasis. Previous clinical trials of efalizumab have demonstrated the efficacy, safety and tolerability of efalizumab for treating moderate to severe chronic plaque psoriasis. The present Phase III trial was designed to further evaluate the safety and efficacy of efalizumab in patients with moderate to severe chronic plaque psoriasis in 54 international centres in Australia, Canada, Europe and Russia. This double-blind, placebo-controlled trial randomised 793 patients in a 2:1 ratio to receive subcutaneous efalizumab 1 mg/kg monotherapy ($n = 529$) or placebo ($n = 264$) weekly for 12 weeks. This included 526 "high need" patients (342 randomized to efalizumab, 184 to placebo) for whom at least 2 systemic therapies were unsuitable due to lack of efficacy, intolerance or contraindication. Efficacy analyses were performed on the intent-to-treat population ($n = 793$); safety analyses were performed on the as-treated population ($n = 793$). Efficacy and safety endpoints are discussed herein; health-related quality-of-life endpoints are discussed in another abstract. At week 12, significantly more efalizumab-treated patients than placebo achieved the primary endpoint, $\geq 75\%$ improvement from baseline in their Psoriasis Area and Severity Index (PASI-75; 31% vs 4%, $P < .001$). At week 12, 54% and 14% of efalizumab-treated and placebo patients, respectively, achieved a 50% PASI improvement PASI. Significantly more efalizumab-treated patients than placebo (27% vs 4%, $P < .001$) achieved a static Physician's Global Assessment of "excellent" or "clear" at week 12, the main secondary endpoint. At week 12, the mean percent PASI improvement was 48% in the efalizumab group (vs 9% placebo, $P < .001$) with statistically significant differences between the treatment groups noted at week 2. Adverse events were generally mild to moderate in severity. The most commonly reported events ($\geq 5\%$ of patients in either group) in efalizumab-treated and placebo patients included headache (26% and 14%), 'influenza-like illness' (10% and 7%), pyrexia (8% and 1%), arthralgia (7% and 3%), rigors (5%, both groups), myalgia (6% and 3%), nasopharyngitis (5% and 4%), and pruritus (4% and 6%). The majority of these events reflect the 'acute adverse events' associated with the first 1 or 2 doses of efalizumab, defined as headache, chills, fever, nausea, vomiting or myalgia occurring within 48 hours of dosing. These results are generally consistent with results from previous efalizumab studies, providing confirmation of the efficacy and safety in patients with psoriasis. Once weekly efalizumab monotherapy results in substantial improvement, demonstrating significant treatment effects on both clinical and quality-of-life measures with an acceptable safety profile, indicating a favourable benefit:risk ratio in adult patients with moderate to severe plaque psoriasis.

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Etanercept Therapy Results in a Sustained Improvement in Skin and Joint Disease in Patients With Psoriatic Arthritis

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Etanercept (Enbrel) has demonstrated clinically significant benefits to patients with psoriatic arthritis (PsA) and psoriasis. Following completion of a 24-week, double-blind, placebo-controlled study of etanercept, 168 patients with PsA and psoriasis (81 originally on placebo; 87 originally on etanercept) enrolled in a 1-year open-label extension study. Psoriasis was evaluated by target lesion score and Dermatologist Static Global Assessment (DSGA) of target lesions, and Psoriasis Area and Severity Index (PASI) for patients with psoriasis $\geqslant$3% body surface area. Arthritis was evaluated according to American College of Rheumatology (ACR) criteria and PsA response criteria (PsARC). To date, total mean exposure to etanercept was 27 weeks for placebo patients and 64 weeks for etanercept patients (maximum 97 weeks). Patients receiving etanercept maintained improvements in both PsA and psoriasis after enrolment in the extension study, while patients originally receiving placebo demonstrated similar improvement after receiving etanercept therapy. At 36 weeks of etanercept therapy ($n = 119$), 49% of patients had "clear" or "almost clear" DSGA, 60% achieved ACR20, 37% achieved ACR50, and 15% achieved ACR70. Of 72 patients evaluable for PASI, 64% achieved a 50% improvement from baseline and 32% achieved a 75% improvement. Etanercept continued to be well tolerated, with no increase in serious adverse events or infections. Etanercept continues to be safe and effective in PsA and provides sustained improvement in skin and joint disease for up to 97 weeks without the need for dose modification.

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Clinical and Pathologic Improvements in Patients with Psoriasis Following Etanercept Monotherapy

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Etanercept (Enbrel), a fully human soluble TNF receptor, significantly improved psoriatic skin disease in patients with psoriatic arthritis. The purpose of this study was to determine the clinical and pathologic improvements following etanercept treatment in a subgroup of patients with psoriasis who underwent a skin biopsy. One hundred and twelve patients with stable chronic psoriasis were enrolled in a multicenter, blinded, randomized 24-week study and received either etanercept 25 mg (57 patients), or placebo (55 patients) by subcutaneous injection twice weekly. The primary efficacy endpoint was 75% improvement in the psoriasis area and severity index (PASI) at 12 weeks. Skin biopsy specimens were taken in a subgroup of 31 patients (17 in the etanercept group, and 14 in the placebo group) at baseline and at week 12. Biopsies were evaluated for epidermal Ki67 and keratin 16 expression, and epidermal thickness. Biopsies from 11 patients were additionally evaluated for total CD3 count and keratinocyte ICAM-1 expression. Baseline disease severity and response to etanercept were similar in the biopsied patients and those in the study as a whole. A significantly higher percentage of patients who received etanercept achieved PASI 75 at 12 weeks (30%) compared with patients who received placebo (2%; $p < 0.0001$). Efficacy continued to improve with longer treatment, with 54% of etanercept-treated patients and 5% of patients who received placebo reaching the PASI 75 at 24 weeks ($p < 0.0001$). Statistically significant improvements in patient global, physician global, and target lesion assessments, and DLQ1 were also observed. Clinical efficacy was mirrored by improvements in biopsy parameters at week 12 compared to baseline. Mean percent reduction in epidermal thickness was significantly greater in the etanercept group versus the placebo group (44% versus 7%, respectively; $p < 0.05$). Reduction in Ki67 and CD3 positive cells, and keratinocyte ICAM-1 and keratin 16 expression was also observed. Enbrel monotherapy was efficacious and well tolerated in the treatment of psoriasis. Clinical efficacy was associated with improvement in several important markers of inflammation including reduction in keratinocyte adhesion protein expression and epidermal T cell infiltration, a decrease in epidermal thickness, and diminished expression of keratinocyte activation and proliferation markers. Inhibition of TNF activity with etanercept may prove useful in the management of psoriasis.

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Etanercept Efficacy Results from an Integrated Multistudy in Patients With Psoriasis

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The purpose was to evaluate etanercept (Enbrel) efficacy in patients ($n = 1187$) with chronic plaque psoriasis by pooling data from a phase 2 and two phase 3 studies. The results from patients who received etanercept 25 mg BIW ($N = 415$) and placebo ($N = 414$), pooled from all 3 studies, and those who received etanercept 50-mg BIW ($N = 358$) and placebo ($N = 359$), pooled from phase 3 studies were compared using a 2-sided Cochran-Mantel-Haenszel test stratified by study. The primary endpoint for all 3 studies was a $\geqslant$75% improvement from baseline in the Psoriasis Area and Severity Index (PASI75) at 12 weeks. A significantly higher proportion of etanercept-treated patients achieved a PASI75 (33% of 25 mg BIW, $p < 0.0001$) at week 12 than placebo, while 49% of those receiving 50 mg BIW etanercept in the 2 studies achieved a PASI75 ($p < 0.0001$). Other measurements, such as physician/dermatologist-reported endpoints including PASI 50 and 90 responses, percentage improvement from baseline in PASI, Dermatologist's Static Global Assessment of clear or almost-clear psoriasis, and subject-reported endpoints, such as the Dermatology Life Quality Index (DLQI) and all DLQI subscales, were significantly better in patients receiving etanercept than in those receiving placebo. A higher degree of clearing at week 12 (PASI90 = 21%) compared with placebo (1%) was seen in patients receiving etanercept 50 mg BIW. There were no serious or significant safety issues appearing in these trials and safety results were consistent with the existing large database of RA patient experience. Results of this integrated analysis demonstrated consistent, robust efficacy of etanercept therapy (25 mg BIW and 50 mg BIW) for chronic plaque psoriasis. A high degree of psoriasis clearing, improvement in both physician- and patient-reported outcomes, and significant impact on quality of life were seen in patients in the studies.

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Age-Related Dermoscopic Prevalence of Clark Nevi

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Background: The dermoscopic classification of Clark nevi (atypical melanocytic nevi) is useful for the examination and the management of individuals with Clark nevi. Objective: To evaluate whether individuals prone a predominant dermoscopic type of Clark nevi in relation to their age. Design: Each lesion was photographed dermoscopically using Molemax (Dermainstruments, Vienna, Austria). All digitized images were evaluated according to the dermoscopic classification of Clark nevi (homogeneous, reticular, globular, homogeneous-reticular, globular-reticular, and globular-homogeneous) and distribution of pigmentation (uniform, central hypopigmented, peripheral hypopigmented, central hyperpigmented, peripheral hypopigmented, and multifocal). Lesions lacking any of these features were classified as not specific. Setting: In a prospective setting individuals were recruited from our pigmented skin lesion clinic in Graz between July 2000 and February 2001. Patients: All consecutive individuals showing more than 10 Clark nevi were subdivided into 10 individuals per 5 designated age groups (0 to 15 years, 15 to 30 years, 31 to 45 years, 46 to 60 years, and individuals older than 60years). During the data-evaluation we additionally invented a sixth age group (summarizing all individuals older than 15 years) that was not included into the primarily study design. Results: A total of 1268 Clark nevi was analyzed. There was a continuously increase of nevi count with age, reaching the maximum in individuals aged from 46 to 60 years, but decreased again in the oldest age group. Among all Clark nevi, the homogeneous-reticular type was most frequent (35.6%), followed by the homogeneous, reticular and globular-homogeneous types (20.8%, 16.4%, 15.8%, respectively), whereas the globular-reticular and globular types were seen in less than 7% and 5%, respectively. Only 2 nevi were classified as not specific. Most of Clark nevi revealed a uniform distribution of pigmentation, followed by a central hyperpigmentation and multifocal pigmentation. An eccentric hyperpigmentation, which is considered to represent an important melanoma simulator, was seen in only 5.4% among all Clark nevi, but in about 30% of Clark nevi from the globular-homogeneous type. Variations on the theme of the globular pattern were the predominant dermoscopic features (globular-homogeneous, globular and reticular-globular type in 36.2%, 24.3% and 21.1%, respectively) in the youngest age group, whereas the homogeneous, the reticular and/or combinations of these two patterns were the predominant types in the adulthood. The probability of the globular type of being presented in individuals younger than 15 years was approximately 70%, but reached 97.8%, 97% and 96.2% for the homogeneous-reticular, homogeneous and reticular types, respectively in individuals aged more than 15 years.

Conclusion: Clark nevi in childhood and young adolescence exhibit variations of the globular patterns, whereas the reticular and/or homogeneous types are the predominant dermoscopic findings in adults. Clark nevi seem to be dynamic lesions that might change their dermoscopic patterns during lifetime from the globular patterns in the early stage of growth to the reticular in their midlife and, the homogeneous patterns at their late stage of growth.

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Palmoplantar Pustulosis – Improvement after Cessation of Smoking

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Ninety-five percent of the patients with palmoplantar pustulosis (PPP) are smokers at the onset of the disease. It is not known if cessation of smoking can improve PPP, and if smoking is the only cause of PPP. Fifty percent of PPP sera produce immuno-fluorescence (IF) of the endothelium from normal palmar skin which might also have a pathogenetic relevance. The aim of this study has been to answer some questions concerning the role of smoking in PPP.

Among 82 patients with PPP the age at the start of PPP was 43 $\pm$ 12 years in those who had started to smoke before 20 years of age and 53 $\pm$ 5 in those starting to smoke after 30 years of age. It was equally common with mild, moderate and severe PPP in current smokers. Four patients had never smoked and had mild PPP and among 13 former smokers only two had severe PPP.

Fifteen patients managed to stop smoking and were examined before and after cessation in parallel with13 who failed or did not want to stop. There was a significant reduction in number of pustules as well as significant decrease in erythema and desquamation in those who stopped smoking whereas there was no improvement in those who continued to smoke. Two patients had a total clearance and some others impressive improvement of previously severe PPP. In another part of the study eight patients who stopped smoking and eight who did not, filled in protocols once weekly with the number of pustules and estimation of the PPP severity on a VAS scale. After three months there was a significant decrease in the number of pustules and of the severity in those who stopped smoking but no improvement in those who did not.

Despite pronounced improvement after smoking cessation, PPP sera gave the same IF of normal palmar endothelium as before cessation of smoking, which indicates that smoking is not the primary cause of PPP. Further studies are needed to identify the underlying abnormality in palmar skin and in particular in the endothelium and the eccrine sweat duct.

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Sera from Patients with Autoimmune Diseases do not have Circulating IgA Antibodies Against Tissue Transglutaminase

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After the identification of tissue transglutaminase (TGc) as the predominant endomysial autoantigen of coeliac disease and dermatitis herpetiformis, diagnostic ELISAs were produced. Sera from patients with a broad spectrum of autoimmune diseases or with non-autoimmune diseases involving enhanced apoptosis, cell lysis and/or putative secondary autoimmune processes showed false positive reactions in these assays, as detected by a number of investigators. Sera from 605 patients and controls were tested in a human TGc ELISA including 304 patients with autoimmune and 122 with other diseases. Anti-gliadin and anti-endomysium antibodies were also determined in 70 patients chosen at random.

Overall 49% of all autoimmune sera were false positive in the human TGc ELISA. The difference between the median titers of the autoimmune and the control sera was significant in every case except for the pemphigus vulgaris sera (bullous pemphigoid, $p = 0.012$; pemphigus vulgaris, $p = 0.152$; SLE, $p = 0.0005$; antiphospholipid syndrome, $p = 0.0002$; for each of the other groups $p < 0.0001$). Significant differences were also found in serum titers between controls and patients with hepatitis C ($p < 0.0001$), psoriatic arthritis ($p = 0.0065$), and malignancies ($p = 0.0005$). Altogether 25% of the sera from patients with hepatitis C, psoriatic arthritis, and malignancies were positive in the human TGc ELISA. In contrast, testing 70 of the false positive patients for total serum IgA levels, AGA and EMA reactivity as well as immunoblots showed that no association of these conditions with gluten sensitive enteropathy can be confirmed. However, further purification of the TGc protein preparation used for coating and elevation of ionic strength in the buffers could eliminate false positive signals.

The currently used method for TGc ELISA allows nonspecific positivity of certain sera from patients having circulating IgA of high affinity against impurities. Thus it is evident that positivity of a TGc ELISA should not alone be taken as the basis for a diagnosis of coeliac disease or dermatitis herpetiformis.

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Hepatitis-C-Treatment Associated Dermatitis as an Example of Iatrogenic Eczema

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Objective Characterization of eczematous dermatitis associated with combination treatment of hepatitis C with peg-interferon-alfa and Ribavirin.Background

In a previous survey of 52 patients undergoing combination treatment, we have found *de novo* skin lesions in 23% of patients. The clinical features of this adverse phenomenon remain to be defined.

Materials and Methods. A dermatologic outpatient structure for prompt referral of patients presenting with cutaneous symptoms during combination treatment for hepatitis C was created. Clinical assessment was completed by allergologic and histopathologic analyses in selected cases.

Results. Overall, 36 patients were referred. All patients complained of pruritus and dry skin. Eczema was the most frequent diagnosis (33 cases), and confirmed by skin biopsy in 5 cases. Most cases presented with diffuse, generalised dermatitis, while 5 patients had nummular eczema, and 3 eczema craquelé. Sequential analyses of total and specific IgE levels in 8 patients showed no correlation with combination treatment. A positive atopy patch test result was found in 5 (of 22 tested) patients; in four of them, the test became negative when repeated after the end of combination treatment. Most patients responded well to topical anti-inflammatory and emollient therapy, and none had to abandon his combination treatment. One case with acantholytic dermatitis, and one case with porphyria cutanea tarda required dose reductions. After the end of combination treatment, cutaneous symptoms resolved in all except two patients.

Discussion. Our observations suggest that the cutaneous side effects of combination treatment for hepatitis C are clinically homogenous, with pruritus, xerosis, and eczematous lesions as key features. Perception of hepatitis-C-associated dermatitis as an iatrogenic eczema may facilitate implementation of preventive and curative measures, and improve compliance with long-term combination treatment regimens. The observed modulation of the atopy patch test result by combination therapy may reflect an immunomodulatory pathomechanism, that warrants further investigation.

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Limb Worn Digital Accelerometers used as Quantifiers of Disease Activity in Pruritic Conditions

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Our group has previously reported work to demonstrate that digital accelerometers can be used to monitor limb activity in children with atopic dermatitis and that the measurements act as a proxy for itch. The method was successfully validated against infrared videoing of subjects at night. These initial studies were confined to children with atopic dermatitis between the ages of 2 and 4. In the present study we report on the wider applicability of this measure in a larger group of children and in adults with a range of pruritic conditions. We have studied 112 participants: 34 adults, age 20–87 (median 50.5) 19 of whom had eczema, 5 liver disease and 10 with a range of other disorders including leukaemia and urticaria pigmentosa. The control adult group comprised 30 healthy adults age 27–74 (median 38.) A further 29 children, age 2–13 (median 6) with 28 with atopic dermatitis and 1 with lichen planus were studied, along with 20 healthy child controls age 2–15 (median 10.5.) There were obvious differences between cases and controls for adults with activity medians approximately twice as high in cases as controls ($p < 0.01$.) Similar 2–3 fold variations were seen for children between active cases and controls ($p < 0.01$.) Absolute scores for cases were 30% higher in adults than children. The relation between subjective measures, SCORAD scores and objective recorded activity was imperfect with much variation unaccounted for – in particular, in adults but not children we found a negative correlation between objective movement activity and self reported quality of sleep. We have studied the temporal pattern throughout he night, and also observed night-to-night variation. Variation occurred from night to night but there was still an obvious person (or disease state) factor effect. These analyses will inform use of the measure in clinical trials.

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Dermoscopic Classification and Follow-up of Halo Nevi

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Halo nevi (HN) are melanocytic nevi surrounded by a depigmented area. These nevi – also termed Sutton's nevi – are characterized by a typical course.

We carried out a dermoscopic classification of HN and studied their changes in size during a follow-up period.

Moreover we investigated regression in melanoma in comparison with HN.

In a retrospective study we classified digital dermoscopic images of HN according to main dermoscopic structural patterns. Images were selected from a database containing 29 383 digital dermoscopic images of 6079 patients.

Whenever we had a record of follow-up digital dermoscopic images of a HN we calculated the percentage of the changes in size of the nevus and the halo component during the follow-up period.

Additionally we studied digital dermoscopic images from the same database of histopathologically confirmed melanoma concerning signs and localization of regression.

We classified 141 halo nevi on 88 patients (median age of 22.4 years). The majority (88.7%) of HN was localized on the trunk. The most common dermoscopic structural pattern was the homogeneous-globular (58 nevi, 41.1%), followed by the homogeneous (32 nevi, 22.7%), and the globular pattern (24 nevi, 17%).

We studied the follow-up of 36 HN. During the first six months of the follow-up period the reduction of the area of nevi was 5.2%. For the remaining follow-up period of 25 months (range 4–61 months) the reduction decreased to 2% per month. The analysis of the halo component showed an increase in 11 HN and a reduction in 18 HN.

We classified 382 digital images of melanoma. 214 melanoma had no signs of regression, 100 melanoma had an eccentric, and 66 melanoma had a central regression. In two melanoma we observed an encircling "halo-like" regression.

Melanoma encircled by a "halo-like" regression is rare. Moreover the images of both melanoma in the study displayed all melanoma specific dermoscopic features and therefore could easily be distinguished from HN. 80% of the 141 HN showed a homogeneous and/or globular pattern, which is generally associated with benign pigmented skin tumors. We found a significant higher reduction of the nevus area during the first six months of the follow-up period. Based on those observations we conclude that a digital dermoscopic follow-up of HN longer than six months is not required.

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Protection by Sunscreens Against Ultraviolet-Induced Lupus Erythematosus

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Since photosensitivity is a hallmark of cutaneous and systemic lupus erythematosus (LE), sunscreens may provide a protective measure against the development of disfiguring cutaneous LE lesions. We performed a retrospective evaluation of 66 patients who had undergone diagnostic photoprovocation-testing with a combination of ultraviolet A and B irradiation. Roughly 75 percent of our LE patients showed a positive reaction to the UV-challenge. Only those patients who showed photosensitivity ($n = 52$) were analyzed further. Most of these patients had discoid or tumidus type LE. We found the combined irradiation with UVA and UVB to be most effective in triggering cutaneous LE (98%). In fact, 30% reacted to the combined irradiation only, and neither to UVA or UVB when applied alone, suggesting a synergistic effect of the combination in some patients. In 19% of the patients, both UVA and UVB induced LE lesions. UVA alone induced LE only in 17 patients (33%). UVB induced LE in 48% of the patients. 96% of the patients that had developed LE in the UVA/UVB field were entirely protected against the development of LE or LE-like lesions by the sunscreen. Protected patients showed either no reaction or pigmentation only. Thus, the capacity of the sunscreen in preventing UV-induced LE was higher than the protective action against UV-induced pigmentation. Our data show that modern sunscreens that efficiently protect against both UVA and UVB are very effective in protecting against UV-induced LE. Preliminary data suggest that daily application of high-potency sunscreens also protects patients against UV-induced LE by natural sun-light. Physicians involved in the management of lupus patients should therefore be encouraged to instruct their patients to use high-potency sunscreens.

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Impact of Efalizumab on Health-Related Quality-of-Life Outcomes in Patients with Moderate to Severe Chronic Plaque Psoriasis: Results of the International Randomised, Controlled Phase III Clinical Experience Acquired with Raptiva (CLEAR) Trial

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Psoriasis can have a significant adverse impact on health-related quality of life (HRQL). The impact on HRQL is multifactorial and can be influenced by disease-, patient-, or treatment-related factors. In order to fully appreciate the impact of psoriasis on patients' lives and to assess the effectiveness of psoriasis therapies, patients should be assessed using measures that encompass both physical and psychological measures. Efalizumab is a targeted recombinant humanised monoclonal IgG_1 antibody that inhibits key processes in the pathogenesis of plaque psoriasis. The present Phase III, double-blind, placebo-controlled trial was undertaken to evaluate the impact of efalizumab treatment on HRQL in patients with moderate to severe chronic plaque psoriasis. Patients ($n = 790$) from 54 international centres were randomised in a 2:1 ratio to receive subcutaneous efalizumab 1 mg/kg monotherapy or placebo weekly for 12 weeks. Interim analyses based on 377 patients who received 12 weeks of efalizumab ($n = 252$) or placebo ($n = 125$) are available. Efficacy and safety analyses are reported in an alternate abstract; HRQL analyses are reported herein. The effects of efalizumab on HRQL were assessed using multiple measures: the Dermatology Life Quality Index (DLQI), the Psoriasis Symptom Assessment (PSA), the Short Form 36 questionnaire (SF-36), and a 10-cm visual analogue itching scale. Efalizumab resulted in significantly greater mean (± SD) improvements in each HRQL endpoint following the first 12-week treatment period compared with placebo. The improvement in DLQI, a measure that assesses the impact of psoriasis and treatment on patient's functioning and well-being, for efalizumab-treated and placebo patients, respectively, was 6.2 (±7.7) vs 2.5 (±6.9) ($P < .001$). Improvement in the PSA frequency 5.7 (±6.2) vs 1.8 (±5.8) ($P < .001$) and PSA severity 6.5 (± 6.6) vs 1.6 (± 6.4) ($P < .001$) scores indicated that the frequency and severity of psoriasis symptoms were significantly decreased in the efalizumab-treated patients relative to placebo. Improvement in the itching score was 2.7 (±3.2) vs 0.6 (±2.6) ($P < .001$), corresponding to a 37% vs 10% mean improvement, consistent with the improvement noted on the PSA subscales. Improvement in the SF-36 summary score was 64.1 (±165.0) vs 8.8 (±141.0) ($P = .013$). Quality of life instruments in the efalizumab-treated patients showed improvement in patients' perceptions of their health and of the social and emotional impact of their disease. In summary, once-weekly efalizumab monotherapy demonstrated significant treatment effects on multiple quality of life measures as well as clinical measures with an acceptable safety profile, indicating a favourable benefit:risk ratio in adult patients with moderate to severe plaque psoriasis. The final results for the entire patient cohort from this 12-week treatment period will be presented.

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Do Features of Skin Ageing Differ Between Asian and Caucasian Women?

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Background. Features associated with skin ageing have been extensively investigated in women of different ethnic backgrounds. However, to the best of our knowledge a detailed comparison of the visual features of skin ageing between women of different ethnicity has not yet been performed.

Objectives. Our objectives were (1) to investigate whether the same photographic scales could be used to document and grade the visual features of ageing on the face of both Caucasian and Asian women and if so, (2) to compare their rate of occurrence between the two groups.

Methods. Photographic reference scales documenting visual features associated with ageing such as wrinkles in different locations, pigmented spots and signs of tissue slackening were established from high quality photographs of French women ranging in age from 20 to 80. After confirming that they allowed for acceptable reproducibility of the assessment of different grades of skin ageing, the scales were used to evaluate the visual features of skin ageing on high resolution facial images of 256 Japanese and 281 French women aged from 20 to 80 years old.

Results. Our photographic reference scales allowed for an acceptable strength of agreement (kappa values > 0.55) in the assessment of visual features of facial skin ageing in Japanese and French women.

In both populations, the different grades of severity (from grade 0 to 5) of the features related to pigmented changes, wrinkling and tissue slackening were represented. However, we've confirmed that differences in age of appearance and rate of aggravation of most of the age-related features do exist between Japanese and French women. Wrinkles occur earlier in French women than in Japanese with all the features related to wrinkles visible in women older than 35 in French and 50 in Japanese excepted for wrinkles located on the upper lip. The features related to wrinkles demonstrated a similar pattern of aggravation in both populations with a more pronounced rate of aggravation for furrows between the eyebrows.

Pigmented spots occur earlier and demonstrate a more pronounced aggravation with age in Japanese women than in French with 4,8% of Asian women up to 34 years old with already visible age spots on the cheek (grade > 1) and up to 80% of the women older than 50 with visible age spots. However, only 20% of the oldest Asian women presented with higher grades of severity (grades 4 to 5). Tissue slackening was found to be more pronounced on the naso-labial fold in Japanese women and on the lower part of the face in French.

Our results demonstrate that differences in facial wrinkles and tissue slackening between Caucasian and Japanese women are rather reflected by their rate of occurrence than by their morphological aspect.

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Objective Severity Measures and Quality of Life in Patients with Psoriasis

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Psoriasis is a chronic inflammatory skin disease of uncertain origin with typical clinical and histologic features. Although the extent of skin involvement can be well defined by the means of PASI and other scoring methods, it is unclear how this appearance correlates with the patients' quality of life.

In a prospective study we have obtained clinical data from patients with psoriasis visiting the dermatological department. Patients with chronic plaque-type and guttate psoriasis were included, and additional features like psoriatic arthritis (PsA) and palmoplantar pustulosis were noted. Clinical severity was assessed by PASI, total disease area, involvement of hands and face, type and duration of the disease as well as the number of hospital based treatments, and the use of systemic treatment. The DLQI (Dermatology life quality index) and 2 simple visual analogue scores on health and psoriasis intensity perception were recorded at admission and one week after discharge.

One hundred and twenty one adult patients (74 male 47 female) with a median age of 51 years (range 19 to 85) were included. Mean PASI at admission was 11.9 (range 0.4–56.4) and DLQI 9.0 (range 0–30). Regarding quality of life, subjective symptoms like itching (90%) and feelings of embarresment (63%) were most prevalent. Impairments in personal relationships and sexual difficulties were reported least frequent. Although parts of the DLQI (Items 1,2 and 4) showed a significant correlation with the PASI, the overall DLQI score only showed a very weak correlation with PASI ($r = 0.17$; $p = 0.06$). Regarding determinats of life quality in psoriasis patients, the affected body surface area and presence of arthritis appeared to be significant factors. Treatment resulted in a mean reduction of PASI of 69% ($p < 0.0001$), and quality of life was markedly improved (mean reduction of 4.4 in DLQI). However, both measures correlated poorly.

Our results imply, that both scores measure different aspects of the disease and are not interchangeable in describing the overall severity of Psoriasis. Although the PASI score may well be used as a severity measure in clinical trials, it may be insufficient to reflect the impairment in quality of life in affected patients.

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Imiquimod Treatment of Lentigo Maligna Induces Complete Tumor Regression

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The term lentigo maligna (LM) is used as a synonym for melanoma *in situ* in sun-damaged skin. Five patients (age 67–80years) with 6 biopsy-proven LM (location: face 5x, shoulder 1x) in whom standard surgical therapy was contraindicated were treated with 5% imiquimod (IQ) cream once a day from 5 to 13 weeks. The complete response rate, based on both clinical and histopathological findings for all LM was 100%.

The immunohistological effects of topical IQ, studied at various time points, revealed an inflammatory cell infiltrate composed of T-helper lymphocytes mixed with a significant number of cytotoxic cells and monocytes/macrophages. These results indicate that IQ induces a cytotoxic T-cell-mediated immune response.

The patients have been followed up for 3 to 18 months without evidence of recurrence. IQ, a local immune response modifier appears to be an excellent therapeutic option for carefully selected patients with LM. Close evaluation of patients, including post-therapy histopathological investigation is essential.

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Safe Psoriasis Control: Evaluation of a New Approach to Assessing Efficacy of Psoriasis Treatments

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Psoriasis is a chronic, incurable disease marked by an unpredictable pattern of remission and spontaneous relapse. Responses to current therapies can be unpredictable, and safety concerns may limit the long-term use of the currently approved systemic therapies and phototherapy. There are several new agents in clinical development, and it is sometimes difficult to determine their true therapeutic benefit-risk. The US Food and Drug Administration and the European Agency for the Evaluation of Medicinal Products efficacy benchmark is the proportion of patients achieving a 75% reduction in Psoriasis Area and Severity Index (PASI-75). Although useful, PASI-75 is a relative endpoint that does not reflect the actual control of psoriasis nor the fact that response to therapy is a continuous variable. Furthermore, PASI-75 as a sole indicator of treatment benefit ignores information such as quality-of-life improvements as well as safety and tolerance of medication. A comprehensive approach that evaluates overall disease control, including physician, patient, and safety assessments, would be a more robust way of estimating the true benefit-risk ratio of novel psoriasis therapies. Efalizumab, an antibody that modulates various T-cell–mediated events underlying the pathogenesis of psoriasis, has been studied in clinical trials including >3000 patients (Phase III trials) and has demonstrated superiority over placebo. Data from efalizumab trials have been further analysed to examine the distributions of endpoints and the relationship between these parameters; this confirmed that physician-measured (eg, PASI) and quality-of-life (Dermatology Life Quality Index; DLQI) outcomes are two separate components of efficacy providing complementary information about the benefit of therapy. The anchor-based method was used to calculate the absolute PASI and DLQI scores, based on Patient's Global Psoriasis Assessment (PGPA) category, required to reflect control of the disease. Effect size was used to validate the cut-off values of PASI and DLQI for the control level. Safety assessments were combined with the efficacy measures, generating a new Good Safe Control approach defined as:

PASI $\leqslant 8$, and DLQI $\leqslant 6$, and no serious adverse events, and no severe AE related to study drug, and not withdrawn.

As an additional validation, results generated using the anchor-based method were compared to cut-off levels presented in external papers. Safe Control results were determined for the first 12-week treatment period of Phase III efalizumab trials. Good Safe Control was achieved in 39% of patients treated with efalizumab 1.0 mg/kg/wk ($n=763$) compared with 10% of patients receiving placebo ($n=479$). The Safe Control approach allows a comprehensive evaluation of a new treatment's benefit-risk profile, and the stringent levels of control take into account the multidimensional aspects of psoriasis and the inter- and intra-patient variability associated with responses to treatment.

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Toxic Epidermal Necrolysis/Stevens Johnson Syndrome: Positive Outcome after Treatment with Dexamethasone Pulse Therapy

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To reduce mortality in toxic epidermal necrolysis (TEN) and Stevens Johnson syndrome (SJS), short courses of high dosed corticosteroids at an early stage of the disease appear to be a rational option in order to influence the immune system that leads to apoptosis and necrolysis. TEN and SJS are acute, rare and life-threatening, most often drug-induced mucocutaneous reactions, belonging to the same spectrum. SJS may progress to TEN, both can be caused by the same type of drugs. Reported mortality rates vary, but are generally high (up to 30% or more). In TEN/SJS epidermal necrosis and detachment result in loss of skin barrier function. Consequently, shock and sepsis are feared complications and the main cause of death.

Besides elimination of the offending drug and maintaining specific intensive care procedures, we applied dexamethasone pulse therapy (DPT), consisting of one i.v. dose of 1,5 mg/kg during three consecutive days. We included 12 consecutive patients with TEN/SJS in this study. Diagnosis was based on the classification by Bastuji-Garin *et al* in 1993. The average severity score (SCORTEN) was 2.5, varying from 0 to 5, median 2. Based on the body surface area (BSA) with detachment, we classified 1 patient as SJS, 7 as TEN and 4 as SJS/TEN-overlap. As soon as the diagnosis was established, we started DPT.

Within 5 days (average 3 days) the progression of the BSA with detachment was arrested in all patients. In all but two cases cutaneous and mucosal lesions healed within 3 weeks. In those 2 patients healing was prolonged, probably due to disseminated cutaneous HSV-infection. One of them also had pre-existent burn cicatrisation.

DPT in combination with specific intensive care treatment appears to promote a positive outcome in SJS/TEN. Compared to mortality prediction according to the SCORTEN-score our results were significantly better.

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Safety of Extended and Long-Term Efalizumab Therapy for Plaque Psoriasis: Results from Phase III Clinical Trials

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The average age at onset of psoriasis is the late 20s, and the disease typically follows a waxing and waning course. Psoriasis is principally an immune-mediated disorder that involves sustained activation of T cells in the dermis and epidermis. Traditional systemic therapies and phototherapy for moderate to severe plaque psoriasis, although effective, all have toxicity issues that affect other organ systems, thus limiting their long-term use or requiring intensive ongoing laboratory monitoring. Because psoriasis is a chronic condition usually requiring treatment over many decades, agents that are safe and effective for long-term use are needed. Efalizumab is a recombinant humanised IgG_1 monoclonal antibody that targets the T-cell functions associated with the pathogenesis of psoriasis. It binds to the CD11a site on the leukocyte function–associated antigen (LFA)-1 expressed on T cells, and prevents LFA-1 from binding to intercellular adhesion molecule (ICAM)-1 on antigen-presenting cells, endothelium, and keratinocytes, thus inhibiting T-cell activation, trafficking into psoriatic skin, and reactivation therein. In two Phase III trials, extended treatment with efalizumab over 24 weeks (Weeks 1–12 were placebo-controlled, Weeks 13–24 were open-label) and long-term treatment for up to 36 months (open-label), for which interim 24-month data are available, have been evaluated. Efficacy outcomes for these two trials are presented in a companion poster; safety data will be presented and discussed in this poster. In one of these Phase III clinical trials, subcutaneous injections of efalizumab 1 mg/kg/wk for 24 consecutive weeks was generally safe and well tolerated. Adverse events (AE) observed in $\geqslant 5\%$ of patients during Weeks 13–24 were nonspecific infection (11.1%), headache (6.1%), and arthritis (5.6%). In the other Phase III trial, the ongoing study assessing the safety of efalizumab 1 mg/kg/wk over 36 months, for which 24 months data are available, has shown that long-term therapy with efalizumab is generally well tolerated. The overall incidence of AE did not increase over time, and compared with the first 3-month period, no new types of common ($\geqslant 5\%$ of patients) AE occurred up to Month 24. The incidence of serious AE, AE leading to withdrawal, infection, psoriasis-related AE, and malignancy, remained low and relatively stable during the 24-month treatment period. There was no evidence of cumulative toxicity, hepatotoxicity, or nephrotoxicity. The targeted activity of efalizumab, which is devoid of the global immunosuppressive effects of traditional systemic therapies for this debilitating condition, provides the first treatment for continuous long-term treatment of patients with moderate to severe chronic plaque psoriasis without increasing toxicity over time.

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Thermographic Analysis of Psoriatic Skin Lesions

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Thermal imaging methods gain more and more applications in medicine including evaluation of allergic tests, morphea, chilblains, port wein stains, burn depth, Raynaud's phenomenon, pneumonia development or arthropathy. Psoriasis is characterized by hyperparakeratosis, inflammatory infiltrate and vascular changes. All of these features could influence body temperature measurements. Microvascular abnormalities and inflammation are considered to be of key importance in the development of psoriatic plaques. So the aim of our study was o evaluate usefulness of thermography in estimation of psoriatic lesion activity.

A series of newly admitted in-patients with plaque type psoriasis vulgaris were included in the study. A total of 350 psoriatic lesions were analysed in a 4-week period. Thermocamera ThermaCam Inframetrics 290E was employed in the study. Temperature resolution was 0.1oC, temperature rage, mean and standard deviation were calculated for the examined plaques. Both thermal and visual images of the patients were recorded. Plaque Severity Score (PSS) was introduced for skin lesions evaluation.

We observed an inter-individual variation of temperature measurements between individuals presenting plaques of comparable parameters as regards erythema and induration (ranged from 1 to 4). Severe desquamation, evaluated as 4, seemed to act as a isolator for proper temperature measurement. We also noted that active, progressing lesions demonstrated increased temperature over the plaques themselves and broad surroundings, which did not correspond to the clinically visible shape of the lesions. To the contrary, stable not progressing lesions presented increased temperature virtually only over the plaques themselves and the shape of individual lesions was mostly preserved. No significant differences were noted between temperature measurements over active and stable lesions.

In conclusion, thermography of psoriatic lesions performed before treatment implementation may be very useful in identifying active, progressing lesion which would require a more potent regimen from the very beginning of therapeutic intervention.

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Evaluating Patient Self-Assessment of Melanoma Risk

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Background. Incidence rates of malignant melanomas are growing rapidly in the Western world; however, this cancer is treatable when diagnosed at an early stage of progression. High-risk individuals would therefore benefit from consulting dermatologists more frequently.
Purpose. To evaluate whether patients can use a questionnaire to correctly identify whether or not they belong to a population group that is at high risk of developing melanoma.
Material And Methods. We conducted a case-control study of 404 persons, with 202 persons with diagnosed melanomas building the case group, and 202 controls matched to the cases according to age and gender. To help build a model for assessing melanoma risk, both groups completed a questionnaire asking about skin and eye color, skin type, skin reaction to sun, number of sun burns, sun exposure, personal assessment of skin damage, number of pigmented skin lesions, and number of congenital large lesions. Furthermore, a physician recorded her observations of these items to determine how well patients are able to answer these questions by themselves.
Results. Logistic regression analysis revealed that the three factors "sun damage", "number of lesions" and "skin type" were all independently associated with the melanoma group. There was good agreement between the models built from the patients' and the physician's answers to the questionnaire, as measured by the area under the ROC curve of the logistic regression models (0.71 vs. 0.75, difference not significant). The patients' sensitivity and specificity of risk-group assessment was 63.5% and 70.8%, respectively. For a trichotomous division into high/medium/low risk, the odds ratio of belonging to the melanoma group was 11.68 for the high risk, and 3.42 for the medium risk patients.
Conclusion. Three primary factors that allow patients to individually assess their risk of developing melanoma were identified. This risk assessment can be performed by a simple questionnaire.

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Measuring Physician Reaction to a Decision Support System for the Diagnosis of Pigmeted Skin Lesions

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Background: The early diagnosis of melanoma is difficult even for experienced dermatologists. A decision support system could help physicians by providing a second opinion.
Purpose. To conduct a pilot study that measures how physicians react when a decision support system either supports or contradicts their initial diagnosis.Material And Methods:
52 volunteer dermatologists of varying experience levels participated in the study. 25 lesion images obtained by epiluminescence microscopy were presented by a computer program that asked the study participants to rate the malignancy of the lesions shown on the images, and to give a dichotomous excise/no excise recommendation. After providing this initial opinion, the decision support system gave its recommendation, and the physicians were given the option to revise their initial opinion. Of the 25 lesion images shown, 14 were pigmented skin lesions, and 11 were cutaneous melanomas at early stages of progression.
Results. Averaging over all 52 study participants and 25 lesion images, there was a change of opinion in 24.51% of the cases when the physician's initial excise recommendation was contradicted by the decision support system. A slight negative correlation between physician experience and susceptibility to following the system's excise recommendation was observed (Kendall tau $= -0.2$, $P = 0.047$). There was no correlation between susceptibility to accepting the system's opinion and the subjective difficulty assessment of the lesion, nor was there a correlation between the physician's susceptibility and whether the system recommendation was benign or malignant. Due to the small sample size, it is difficult to measure whether some physicians are significantly more susceptible than others.
Conclusions. In more than 75% of the cases, the study participants were reluctant to accept the computer system's recommendation. This can be attributed to the fact that the system's characteristics were not made public to the study participants beforehand, and the participants had to develop a "feel" for the system's performance while working with it. Bayesian modelling of the physicians' opinion of the system, and thus his/her confidence in it, will provide a deeper understanding of the results observed in this study.

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Pharmacological and Clinical Activities of 5-Alpha Avocuta : Application for the Management of Androgenic Disorders

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Androgen-dependent disorders, such as seborrhoea, acne and alopecia are among the most common diseases encountered by dermatologists in daily practice. These pathologies are in part related to an hyper-activity of 5-alpha reductase, the enzyme that metabolises testosterone into dihydrotestosterone (DHT) a major potent androgen in human skin. In this work, we have investigated the efficacy of a lipidic fatty ester: 5-alpha Avocuta (butyl avocadate). Butyl avocadate is obtained from refined avocado oil through a biotechnological process, and is purified by molecular distillation.

We have demonstrated using dermal fibroblasts in culture that 5-alphaAvocuta is a potent inhibitors of 5-alpha reductase type I activity (-49% for the smallest dose tested 0.01%).

A multi-centric clinical evaluation, under dermatologist control, was conducted on 27 volunteers. The purpose of this study was to test the efficiency of a shampoo (1% of 5-acute Aocuta) after 3 weeks of application (1 application/2 days). Analysis of sebum secretion (Sebufix® F16) by visual scoring and by image analysis (Skin Visiometer®, SV600, CK, Germany) demonstrate that this shampoo is able to significantly reduce sebaceous production. Clinical investigations by the dermatologist (analogic scale) show that the shampoo clearly improves greasy hair aspect, and is able to reduce itching and pruritus, as well as dandruffs. The auto-evaluation by the volunteers confirms these data. The efficiency of 5-alphaAvocuta, formulated at 2% in a skin care, has been also demonstrated in the management of the hyper-seborrhoea of the face. After 3 weeks (application twice a day), this specific formulation was able to reduce the sebum production (-30%) at the level of the face, to improve all the clinical parameters (-31% in average) and to reduce the number of open comedons. The volunteers have also approved the efficiency of the product (78% good opinion), and they have reported an improvement of their quality of life.

5-alpha Avocuta is a new and original inhibitor of 5-alpha-reductase type I activity with proven efficiency on human scalp and skin disorders related to hyper-seborrhoea. This natural active ingredient may open the way to the formulation of original and efficient products dedicated to the treatment of androgenic disorders.

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Etanercept Improves Quality of Life of Patients with Chronic Plaque Psoriasis: A Meta-Analytic Summary of Randomized Clinical Trials of Etanercept

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In psoriasis, there exists a diverse set of clinical criteria for assessing both severity and the effectiveness of treatment including Quality of Life (QoL). Three randomized, double-blind, placebo-controlled clinical trials (RDBPCTs) have shown separately that etanercept provides significant improvement in psoriatic skin disease and QoL among patients with moderate to severe chronic plaque psoriasis. Using meta-analytic techniques, the efficacy of 2 dose regimens of etanercept (Enbrel) (25 mg or 50 mg twice weekly) compared with placebo in improves QoL as measured by the Dermatology Life Quality Index (DLQI) at 12 weeks. We report efficacy of etanercept in improving QoL in 1187 patients across one phase 2 and two phase 3 RDBPCTs. These studies had similar inclusion and exclusion criteria. Comparisons presented between the 25 mg twice weekly dose ($N = 415$) and placebo ($N = 414$) used summary data pooled across all 3 studies. Comparisons between the 50 mg twice weekly dose ($N = 358$) and placebo ($N = 359$) used summary data pooled across the phase 3 studies. The percentage improvement at week 12 from baseline in DLQI total score and subscales was analyzed using fixed-effects inverse variance meta-analysis. A two-sided Z-test was used at a significance level of 0.01 to test whether etanercept 25 mg or 50 mg twice weekly was statistically significantly different from placebo. The mean baseline values of DLQI were similar at baseline across treatment groups and studies. The mean percentage improvement in DLQI scores from baseline were 51.5% (95% CI: 44.6, 58.1) and 59.1% (95% CI: 51.6, 66.6) for the etanercept 25 mg and 50 mg twice weekly groups respectively compared to placebo ($p < 0.0001$). Further, the percentage improvement from baseline at week 12 for each of the 6 subscales of the DLQI was statistically significantly superior for both etanercept 25 mg and 50mg twice weekly doses. This meta-analysis of the three RDBPCTs confirms etanercept 25 mg and 50 mg twice weekly provided consistent, robust efficacy in improving patients' QoL. Our results are also consistent with the findings of previously reported individual RDBPCTs.

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Clinically Meaningful Improvement in Dermatology Life Quality Index in Patients With Chronic Plaque Psoriasis: A Meta-Analytic Summary of Randomized Clinical Trials of Etanercept

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Three randomized, double-blind, placebo-controlled clinical trials (RDBPCTs) have shown that etanercept (Enbrel) provides Clinically Meaningful Improvement (CMI) in Quality of Life (QoL) among patients with moderate to severe chronic plaque psoriasis. Using meta-analytic techniques, we assessed the efficacy of the 2 dose regimens of etanercept (25 mg and 50 mg twice weekly) compared with placebo in improving QoL as measured by the Dermatology Life Quality Index (DLQI) at 12 weeks. All three RDBPCTs of etanercept were included in the meta-analysis: (1) two-arm phase 2 study of etanercept 25 mg twice weekly ($N = 57$) or placebo ($N = 55$); (2) four-arm phase 3 study of etanercept 25 mg once-weekly ($N = 159$) or twice weekly ($N = 161$), etanercept 50 mg twice weekly ($N = 163$) or placebo ($N = 166$); (3) three-arm phase 3 study of etanercept 25 mg twice weekly ($N = 194$) or 50 mg twice weekly ($N = 194$) or placebo ($N = 193$). These studies had similar inclusion criteria. Subjects had stable plaque psoriasis involving at least 10% body surface area and had received prior systemic or phototherapy, or were considered candidates for systemic or phototherapy. The CMI in DLQI total score was defined as 5 points. Odds of achieving CMI in DLQI total scores were analyzed using the pooled Mantel-Haenszel odds ratio method. A two-sided Z-test was used at a significance level of 0.01 to test whether etanercept 25 mg or 50 mg twice weekly was statistically significantly different from placebo. The mean baseline values of DLQI total were similar across treatment groups and across studies. The pooled Mantel-Haenszel odds ratio for achieving CMI in DLQI was estimated to be 4.20 (95% confidence interval: 3.14, 5.63) and 5.87 (95% confidence interval: 4.25, 8.10) for etanercept 25 mg and 50 mg, twice weekly respectively compared to placebo ($p < 0.0001$). This implies that odds of achieving CMI in DLQI total scores were 4 to 5 times higher for either etanercept 25 mg twice weekly or 50 mg twice weekly compared with placebo. This meta-analysis of three RDBPCTs confirms the efficacy of etanercept in providing CMI in DLQI.

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Efficacy Outcomes During Extended and Long-term Use of Efalizumab in Patients with Moderate to Severe Chronic Plaque Psoriasis

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With the current unmet medical needs for agents that can be safely used continuously in patients with psoriasis, and advances in our understanding concerning psoriasis pathogenesis, a number of targeted biological therapies are in various stages of development and approval for psoriasis. These new therapies are providing physicians and patients with new therapeutic options. Efalizumab, a recombinant humanised monoclonal IgG_1 antibody, is a targeted biological therapy that inhibits the T-cell processes that underlie the pathogenesis of psoriasis, including T-cell activation in the lymph nodes, T-cell trafficking from the circulation into psoriatic skin, and reactivation therein. In one Phase III trial, after 24 weeks of continuous therapy with efalizumab, more patients achieved $\geqslant 75\%$ improvement in their Psoriasis Area and Severity Index (PASI-75) relative to Week 12 (44% vs 27%; 95% CI 38.6%, 49.0%). Mean percent PASI improvement continued to increase through Weeks 13 to 24 to reach 67% after 24 weeks of continuous treatment with efalizumab. In addition, improvements in all health-related quality-of-life (HRQL) measures achieved at Week 12 were maintained through Week 24. An alternate open-label Phase III study is ongoing to assess the efficacy of continuous efalizumab over 36 months. Interim 24-month data are available: the proportion of patients who received up to 24 months of continuous efalizumab therapy and achieved PASI-50, PASI-75, and PASI-90 was 64%, 47%, and 27% (intent-to-treat analysis; $n = 339$), respectively. The results of these 2 trials demonstrate that efalizumab provides continuing PASI improvement through 24 weeks of treatment, and the 24-month results indicate that once-weekly subcutaneous efalizumab can provide continuous control of the symptoms of moderate to severe chronic plaque psoriasis over the long term.

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Clinical and Etiologic Differences Between Toxic Epidermal Necrolysis (TEN) and Generalized Bullous Fixed Drug Eruption (GBFDE)

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In the spectrum of severe adverse skin reactions that present with blister formation toxic epidermal necrolysis (TEN), Stevens-Johnson syndrome (SJS) and generalized bullous fixed drug eruption (GBFDE) are important differential diagnoses. SJS and TEN are acute life-threatening conditions with various amounts of skin detachment and hemorragic erosions of mucous membranes mainly caused by drugs. In contrast, GBFDE is an important differential diagnosis that can be separated by clinical means, but not in terms of the histopathology. GBFDE presents with generalized well demarcated red or brownish plaques with blisters. Mucous membranes may be affected in a mild way; the overall condition of the patient is good. Frequently GBFDE is induced by cotrimoxazole (trimethoprim/sulfamethoxazole), which is also known to induce SJS and TEN, and a previous adverse reaction is often reported to the same drug.

The following analysis was undertaken to elucidate the differentiation between cases of SJS/TEN and GBFDE. Therefore, a re-review of cases using more specific criteria to differentiate between SJS/TEN and GBFDE should allow to detect misclassification of cases.

984 cases of SJS, SJS/TEN overlap and TEN were ascertained by the population-based registry in Germany between 1990 and 1999, of which 131 had taken cotrimoxazole within two weeks before the onset of the adverse reaction. From these cases as well as 38 cases with suspected GBFDE a random sample of 57 cases (44 SJS and TEN, 13 GBFDE) was taken and re-reviewed. In this analysis specific emphasis was given to the time latency between beginning of drug use and onset of the adverse reaction.

For definite cases of SJS or TEN the median between beginning of cotrimoxazole use and onset of the adverse reaction is 9 days (25% quartile 6 days, 75% quartile 14 days). In doubtful cases the time latency is only 2 (2–9). For definite cases of GBFDE the time is one day (1–2) and thus by far shorter.

The high amount of doubtful cases after the re-review reveals the difficulty to apply approved detailed definitions to the variety of clinical patterns of cutaneous adverse reactions. We could confirm a high correlation of time latency between beginning of drug use and onset of SJS and TEN as well as GBFDE for cotrimoxazole. GBFDE should be considered as diagnosis when drug exposure occurs outside the relevant time latency for SJS and TEN, especially in cases with widespread epidermolysis and previous episodes.

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The Relationship Between Patient-Rated Quality of Life and Clinicians' Management Decisions in Psoriasis – A Prospective Study

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The aim of this study was to prospectively determine the relationship between patient-rated quality of life (QOL) and clinicians' management decisions in psoriasis, in current routine clinical practice. Between July 2003 and March 2004 (35 weeks), all 687 outpatient consultations for psoriasis management, by 13 clinicians, at the Welsh Institute of Dermatology, University Hospital of Wales, Cardiff were included in the study. Patients aged 18 years or older were posted a Dermatology Life Quality Index (DLQI) questionnaire that they received the day after their consultation. This ensured that the DLQI scores were not available to the clinicians during the consultations. The consenting patients were advised to complete the DLQI questionnaire that relates to the week preceding completion, within 2 days of the consultation, ensuring an accurate assessment of the QOL of each patient at the time management decisions were taken. Case notes were examined and the main management decision taken for each patient was assigned to one of eleven categories. 383 (55.7%) completed DLQI questionnaires were included in the analysis of data as follows: No change to the main therapy type despite an increase in potency, dose or frequency of that therapy [MD1a](n = 29, DLQI range 1–29, DLQI mean ± standard deviation 11.9 ± 8.1); No change to main therapy at all [MD1b](n = 199, 0–3, 8.9 ± 7.2); no change to the main therapy type despite a decrease in potency, dose or frequency of that therapy [MD1c](n = 27, 0–21, 6.2 ± 5.8); change from one type of topical to another type of topical therapy [MD2](n = 49, 1–7, 11.2 ± 6.7); topical to systemic therapy [MD3](n = 14, 3–30, 14.8 ± 6.9); one type of systemic to another type of systemic therapy [MD4](n = 3, 9–27, 16.7 ± 9.3); start photo(chemo)therapy [MD5](n = 34, 2–28, 13 ± 7.4); plan admission to hospital [MD6](n = 9, 5–21, 14.4 ± 5.0); refer to dermatology day treatment unit [MD7](n = 9, 3–20, 8.6 ± 6.6); discharge from specialist dermatology to primary care [MD8](n = 7, 1–13, 4.0 ± 4.2). Statistically significant differences were seen between the mean DLQI scores for MDs 1a & 1b, 6 & 7, ($p < 0.05$), 1a & 1c, 1a & 1b, 5 & 1b, 6 & 1b, 2 & 8, 3 & 8, 5 & 8, 6 & 8 ($p < 0.01$) and 5 & 1c, 6 & 1c ($p < 0.001$) using the Mann-Whitney U test. There was a striking difference in mean DLQI scores between patients in whom there was no change to therapy at all (MD1b) and in whom major new interventions were taken i.e. MDs 3 + 4 + 5 + 6 (n = 60, DLQI mean ± standard deviation 13.8 ± 6.9, median 13, $p = 0.00007$). The median DLQI scores seen in decisions MD 1a (13), MD 3 (14.5), MD4 (14), MD5 (11) and MD6 (13) are consistent with the proposed DLQI banding of 11–20 indicating a "very large effect" on overall QOL. The median DLQI scores seen in MD1b (7), MD7 (7) and MD2 (10) are consistent with banding 6–10 indicating a "moderate effect", and low scores in MD1c (4) and MD8 (3) consistent with banding 2–5 indicating only a "small effect" on patient's overall QOL. This study demonstrates an overall relationship between patient-rated QOL and the nature of management decisions taken in psoriasis. The wide score scatter for each decision seen in this study indicates that clinicians' management decisions may not have been appropriate in some patients. This study also highlights the importance of including formal QOL assessments in addition to physical measures when clinical decisions are taken over psoriasis management, for example, the use of systemic and new potent biological therapy, with its potential side effects. The initial data from this study are important in developing such criteria to aid clinicians make management decisions tailored to each individual patient.

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The Farnsworth panel D 15 test – a Simple Instrument to Detect High Risk Melanoma Patients? First Results of a Study Including 300 Patients with Malignant Melanoma

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Melanoma-associated retinopathy (MAR) is a paraneoplastic syndrome in patients with malignant melanoma, that is rarely seen with all its clinical signs and symptoms. The pathomechanism is supposed to result from antibody production against melanoma antigens which cross-react with retinal epitopes. Beside light sensations, night blindness and visual loss, color loss for blue (tritan dysfunction) has been reported. The onset of MAR symptoms is correlated with a rapid progress of disease and a worse prognosis for progress-free and overall survival. Recently performed ophthalomological examinations as well as immuno-fluorescence assays on retinal tissue using autologous serum samples of melanoma patients could prove, that subclinical signs as well as the presence of antiretinal antibodies is more frequent than supposed. As intensive ophthalmological examinations and indirect immuno-fluorescence on retinal slides are time-consuming and cost-intensive they usually cannot be performed in melanoma aftercare. The Farnsworth panel D 15 test is a color test which is able to detect all kinds of color deficiencies. Patients have to arrange 15 different color caps using a blue reference cap. Mapping of the color sequence in a diagram shows the existence of color loss. This study including 300 melanoma patients (AJCC stage 0: n = 3; stage I: n = 149; stage II: n = 53; stage III: n = 56; stage IV: n = 39) and 100 healthy controls was performed to answer the question if the Farnsworth panel D 15 test alone is able to detect high risk melanoma patients. 26% of all melanoma patients showed tritan dysfunction, this percentage was much higher than in the control group (2%). The presence of tritan dysfunction correlated with stage of disease, tumor thickness, Clark Level and age, but not with sex, S-100 value or time since first diagnosis. Actually, follow-up examinations are performed and compared with immunofluorescence experiments with autologous serum on retinal tissue. After this evaluation is should be possible to state if the Farnsworth panel D 15 test is a simple instrument to detect high risk melanoma patients in melanoma aftercare.

414 [Oral 004]

Psoriasin (S100A7) Protects Human Skin from *E. coli*-Infection *In Vivo*

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Human skin is potentially exposed to a variety of microorganisms but surprisingly resistant towards the gut bacterium *E. coli*. Recently we identified psoriasin as a major *E. coli* killing S100 protein from healthy human stratum corneum extracts. We could demonstrate that the antimicrobial activity of psoriasin is inhibited by Zn^{2+} and that psoriasin-mRNA is inducible in primary keratinocytes by proinflammatory cytokines and contact with bacteria. This study was initiated to investigate the relevance of psoriasin as antimicrobial protein *in vivo*.

Immunohistochemical analysis of healthy human skin derived from different body sites using an anti-psoriasin antibody reveals a focal expression pattern mainly at locations with a high microbial colonisation rate suggesting a local induction and release. To prove this hypothesis we artificially applied *E. coli* ATCC-No. 35218 on the forearms of healthy volunteers (n = 18) and were able to show a rapid and high effective killing of the bacteria. To investigate whether psoriasin is responsible for *E. coli*-killing we developed neutralizing monoclonal anti-psoriasin antibodies and could show that the monoclonal antibody HL15-4 - but not the heat-inactivated moAb- inhibits *in vitro* the *E. coli*-killing activity of psoriasin. Pretreatment of the forearm skin with the antibody before experimental application of *E. coli* led to a dose-dependent increase of bacterial survival (n = 5). Using a sandwich-ELISA we could identify significant bactericidal levels of psoriasin in washing fluids derived from the forearm skin in all persons. The psoriasin amounts present at the skin surface varied depending on the location.

Our findings show that human skin secretes *in vivo* in a donor and site-specific fashion the S100 protein psoriasin as principal *E. coli*-killing antimicrobial compound explaining the unexpected resistance of skin towards *E. coli*, despite its high abundance in the daily life.

415 [Oral 024]

Papillomavirus-like Particles (VLP) Carrying HPV16-L2 Peptides Induce Cross-Neutralizing Antibodies to Genital HPV 11

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Vaccination in human trials with papillomavirus-like particles (VLP) of types 16 and 18 has shown 100% efficiency conferring type-specific protection against these most prevalent HPV infections and associated neoplasia. However, protection against 13 high-risk HPV types accounting for additional 30% of cervical cancers remains a challenge. Although peptides of HPV16 L2 minor capsid protein have been identified that induce cross-neutralizing antibodies to HPV6, 11 and 18, L2 is poorly immunogenic in the context of L1/L2 VLP. Thus the aim of this study is to display HPV16L2 epitopes on immunogenic surface loops of L1-VLP, capable of inducing a strong anti-L2 antibody response with cross-neutralizing activity to other mucosal types. Two previously described HPV16L2 epitopes (representing amino acids (aa) 69–81 (A) and 108–120 (B)) were engineered into L1 of bovine PV type 1 (BPV1) (between aa residues 133/134). Chimeric proteins were expressed in insect cells, purified on density gradients, and antigenicity was verified by Western blot. Electron micrographs demonstrated, that chimera B self-assembled efficiently into capsomeric VLP similar to wt L1, whereas for chimera A mainly pentamers or aggregates thereof were observed. Immunization of NZW rabbits using Freunds adjuvant induced antisera that recognized GST-HPV16L2 with a titer of 1,000–10,000 by ELISA. Importantly, when examined in an RT-PCR transient infectivity assay using native HPV 11 virions, antisera induced by chimeric protein A, but not B, neutralized this distantly related type. Induction of cross-neutralizing antibodies by chimeric L1/L2 VLP may facilitate the generation of broad-spectrum vaccines that protect against a majority of relevant mucosal HPV and associated neoplasia.

416 [Oral 026]

DC-SIGN-Mediated Infectious Synapse Formation Enhances Transfer of HIV Infection from Dendritic Cells to T Cells

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Dendritic cells (DCs) expressing the DC-specific C-type lectin DC-SIGN capture small amounts of HIV on mucosal surfaces and facilitate viral transmission to $CD4^+$ T cells in lymph nodes, where massive viral replication occurs. In order to analyze the role of DC-SIGN in early events of HIV infection and prevent HIV transmission from DCs to T cells, we disrupted DC-SIGN function through lentiviral-mediated RNA interference. By suppressing DC-SIGN expression, we demonstrate that DC-SIGN is a major attachment factor for HIV and the mycobacterial cell wall component ManLAM on human DCs. Furthermore, DC-SIGN suppression in DCs impairs infectious synapse formation between DCs and $CD4^+$ T cells. DC-SIGN-negative DCs are also unable to enhance transfer of HIV infectivity of T cells *in trans*. In conclusion, we show that DC-SIGN is required for infectious synapse formation between DCs and T cells which enables *trans*-enhancement of HIV infection to T cells.

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***C. albicans* Activates Human Primary Endothelial Cells by IKK2/IκBα/NF-κB- and p38-MAP Kinase-Dependent Intracellular Signalling Pathways**

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Endothelial cells are not only physical barriers between different compartments but are also capable of actively regulating defence mechanisms against microbial pathogens such as *Candida*. We here analyzed the effects of *C. albicans* (strain SC5314) on different intracellular signalling pathways which mediate the expression of proinflammatory cytokines, chemokines and antimicrobial peptides by endothelial cells. Co-culture of *C. albicans* blastospores with human umbilical vein endothelial cells (HUVEC) at a MOI of 1 resulted in a significant up-regulation of IL-8 and ICAM-1 expression. Immune complex kinase assays revealed that *C. albicans* induced activation of IKK2 which is followed by degradation of IκBα as determined by Western blot analysis. However, activation of the NF-κB signalling module appears to be retarded when compared to activation by TNF-α. To assess the functional relevance of activation we studied endothelial cells which were retrovirally infected to express a dominant-negative mutant of IKK2, i.e. IKK2KD. Co-culture of such cells with *C. albicans* blastospores no longer resulted in up-regulation of IL-8 and ICAM-1 expression thus confirming the requirement of NF-κB activation. Another signalling pathway identified to be activated by *C. albicans* is the p38 mitogen-activated protein (MAP) kinase pathway. We here demonstrated activation of p38 by immune complex kinase assay and verified functionality with respect to IL-8 expression by the use of a highly specific inhibitor. In conclusion, our data demonstrate that *C. albicans* activates distinct intracellular signalling pathways in human endothelial cells which are necessary for the expression of molecules apparently playing prominent roles in the pathophysiology of *Candida* infections.

418 [Oral 029]

DC-Derived Antagonistic IL-12 Homodimer Induces Th2-Immunity in Cutaneous Leishmaniasis

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IL-12 release of infected dendritic cells (DC) is critically involved in Th1-education in various disease models. In cutaneous leishmaniasis, protection in C57BL/6 mice is the result of Th1-immunity, whereas susceptible BALB/c mice develop Th2-responses. Bioactive IL-12p70 is a heterodimer composed of p35 and p40, whereas p40 released in excess can be found in the inactive p40 monomeric form or as (p40)2 homodimer with inhibitory activity. The ratio between p40/(p40)2 is regulated by IL-4 *in vivo*. In this study, we analysed differences in the release of IL-12 in more detail. In the presence of IL-4, activated bone marrow-derived DC (generated with GM-CSF) from both resistant and susceptible mice released comparable amounts of IL-12p70 and p40 confirming previous findings. However, without IL-4 (reflecting more physiological conditions during DC activation *in vivo*), BALB/c-derived DC released significantly more p40 than C57BL/6-DC as detected by ELISA. We next determined, whether IL-12 was released as monomeric p40 or in the form of the natural inhibitor (p40)2. Interestingly, in western blot analyses of both C57BL/6 and BALB/c DC-supernatants a large proportion of anti-IL-12p40 reactivity was detected at 80kDa. We found substantially more (p40)2 in BALB/c DC-supernatants. To determine the physiological role of IL-12(p40)2 in cutaneous leishmaniasis, C57BL/6 and BALB/c mice were treated locally with 1 μg recombinant (p40)2 or PBS intradermally during T-cell priming (days 1–3 post infection with $2 \times 10E5$ L. major). Lesions in (p40)2-treated groups were significantly larger in both mouse strains compared to controls from wk 3 on (e.g. BALB/c mice: 125 ± 20 vs. 63 ± 16 mmE3 at wk 4, $n = 7$, $p < 0.01$). In parallel, lesional parasite loads were $\sim$ 2-log higher after (p40)2-treatment and the cytokine profile at wk3 was shifted towards a Th2 profile. In summary, genetical differences in the production of IL-12p40 homodimer from infected DC may contribute to disease outcome in cutaneous leishmaniasis.

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Interferon Resistance Promotes Oncolysis by Influenza Virus NS1-Deletion Mutants

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The NS1 protein of influenza virus is a virulence factor that counteracts type I interferon (IFN)-mediated antiviral response by the host. A recombinant influenza A virus which lacks the NS1 protein only replicates efficiently in systems that contain defective IFN pathways. In this contribution we demonstrate that the conditional replication properties of NS1-modified influenza A virus mutants can be exploited for the virus-mediated oncolysis of IFN-resistant tumor cells. IFN resistance in analyzed tumor cell lines correlated with a reduced expression of STAT1. Addition of exogenous IFN or supernatant of virus-infected endothelial cells inhibited viral oncolysis in IFN-sensitive but not in IFN-resistant cell lines. The oncolytic potential of NS1-modified influenza A virus mutants could be exploited *in vivo* in a SCID mouse model of a subcutaneously implanted human IFN-resistant melanoma. The data indicate that IFN-resistant tumors are a suitable target for oncolysis induced by NS1-modified influenza virus mutants. STAT1 might serve as a marker to identify these IFN-resistant tumors.

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An Infectious Endogenous Retrovirus Derived from Human Melanoma Cells

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We show that human melanoma cells produce retrovirus-like particles that exhibit reverse-transcriptase activity, package sequences homologous to human endogenous retrovirus K (HERV-K), and contain mature forms of the Gag and Env proteins. We also demonstrate expression of the *pol* gene and of Gag, Env and Rec proteins in human melanomas and metastases but not in melanocytes or normal lymph nodes. The melanoma-derived particles are infectious for Madin-Darby bovine kidney cells. The data suggest that expression of retroviral genes and production of infectious retroviral particles is activated during development of melanoma.

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Consistent Human Papillomavirus Infections in Different Non-Melanoma Skin Cancer and their Recurrences

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Objective: Ultraviolet radiation is the major risk factor of non-melanoma skin cancer (NMSC) and cutaneous HPV types seems to play a role during skin carcinogenesis. We examined the presence of cutaneous HPV and their variants in NMSC and their recurrences.
Patients and Methods. Twenty-four biopsies of 4 patients with invasive NMSC were analyzed by a HPV L1 PCR-based method to detect all 24 cutaneous HPVs of the B1 and B2 group followed by typing using reverse line blotting. In a retrospective study with prospective value recurrent NMSC were followed up between 8 and 37 months. HPV typing of selected cases were verified by E6 type-specific PCR followed by primer cycle sequencing (HPV 8, #184–656; HPV 14, #218–325; HPV 21, #209–432; HPV 36, #210–316).
Results. Consistent single and multiple HPV infections were detected in 2 of 4 patients. All 7 NMSC of one immunosuppressed patient developed at sun-exposed sites after 4, 8, 9, 11, and 37 months were infected with HPV 21 and HPV 36 until 11 months of follow-up. The HPV 36 E6 variant 311T was detected until 9 months of follow-up and the metastatic squamous cell carcinoma (SCC) developed after 11 months was infected with the E6 variant [311T, 261C (L21S)]. The same HPV 21 E6 variant [226C, Del (244–273, AA16–25)] was identified in the primary SCC and recurrences after 4, 8, 9, and 11 months. In another immunocompetent patient both the primary SCC and the metastasis developed after 8 months were infected with HPV 14. Squamous cell carcinomas of two non-immunosuppressed patients showed no consistent HPV infections. One of three and 7 of 9 SCC were HPV negative.
Conclusions. Consistent HPV infections in invasive SCC and their recurrences suggest a role of cutaneous HPV during skin carcinogenesis in a subset of NMSC. The spatial localization of HPV will be examined.

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After Challenge with *Leishmania major*, Strain-Dependent Differences in Inflammatory Immune Responses are Most Prominent at the Site of Infection

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Healing in cutaneous leishmaniasis is associated with IL-12-dependent IFN-γ-producing CD4/Th1 cells as observed in resistant C57BL/6 mice. In contrast, susceptible BALB/c mice succumb to infection because of Th2 immunity which fails to eliminate intracellular parasites. During physiological low dose infections with *Leishmania major* protection by $CD8^+$/Tc1-cells in C57BL/6 mice was also achieved. We described previously that fusion proteins of HIV-1 TAT and *Leishmania*-antigen LACK efficaciously vaccinate against progressive disease by facilitating MHC class I-dependent induction of *Leishmania*-specific $CD8^+$ T-cells. Thus, activation of CTL may be beneficial for protective immunity. In contrast to C57BL/6, the physiological role of $CD8^+$ T-cells in BALB/c mice during leishmaniasis is not known yet. To do this, we analyzed the inflammatory infiltrate of infected ears as well as draining lymph nodes (LN). Infections were initiated using $2x10^5$ infectious-stage promastigotes. Infected ears and LN were collected weekly and inflammatory cells were characterized by FACS. In LN as well as dermis of BALB/c mice significantly more total cells were found than in C57BL/6 mice correlating to increased lesion volumes. In dermal lesions, no strain-dependent differences regarding recruitment of $CD4^+$ and $CD8^+$ T-cells were observed, whereas BALB/c showed a 2-fold increase of $CD4^+$ cells in LN compared to C57BL/6 mice. The number of $CD4^+$ cells was 2-fold higher than of $CD8^+$ cells independent of the strain studied and immigration of lesional $CD8^+$ T-cells was delayed compared to $CD4^+$ cells (wk3 vs. wk5). Interestingly, significant increases (3-fold) were found in the number of lesional neutrophils and $CD11c^+$ DC in BALB/c mice suggesting that DC alone are not sufficient for protection. In summary, susceptibility of BALB/c mice against infection with *Leishmania major* is not caused by impaired recruitment of lesional T-cells. Most strain-specific differences were found in dermal lesions suggesting that relevant immune responses occur directly at the site of infection.

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Expression Profiling Reveals Sets of Genes Regulated Differentially in HaCaT Keratinocytes Following Exposure to Secreted *Pseudomonas* Products

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We have shown previously, that the skin is able to defend infection by constitutive and upregulated production of antimicrobial peptides and that the extent of microbial induced keratinocyte defense gene expression also depends on microbial strains and growth conditions. For a better understanding of the mechanisms in the hostile interaction between keratinocytes and *Pseudomonas aeruginosa* and to determine the parameters for successful protection against infection we compared alterations of (HaCaT) keratinocyte gene expression upon exposure to supernatants of *P. aeruginosa* differing in their ability to induce expression of the antimicrobial peptide hBD2.

Differentially expressed keratinocyte genes were identified using an inflammation specific DNA array. The results were validated using real-time (kinetic) reverse transcription-polymerase chain reaction (RT-PCR). From 376 genes present on the inflammation specific DNA-array 36 were identified to differ in keratinocyte expression by a factor of at least 2 following stimulation. The identified genes could be grouped according to their expression pattern in genes upregulated following stimulation with the hBD2-inducing *P. aeruginosa* supernatant ("Set 1": e.g. Psoriasin, Calgranulin), genes upregulated following stimulation with a mixture of hBD2 inducing and hBD2 not inducing *P. aeruginosa* supernatant ("Set 2": e.g. MCP1, IL-8) and in genes down regulated following stimulation with this *P. aeruginosa* supernatant mixture ("Set 3": Integrin ß4, HSPB1).

Depending on strain and growth conditions, secreted *Pseudomonas factors* obviously include factors which induce or inhibit keratinocyte expression of antimicrobial peptides. Upregulation of genes like MCP1 or IL-8 following keratinocyte stimulation with a mixture of hBD2 inducing and not-inducing *Pseudomonas* supernatants (which results in reduced expression of antimicrobial peptides) indicates that keratinocytes are able to activate additional defense mechanisms under conditions restricting local innate defense mechanisms. Future experiments will focus on the identification of those *P. aeruginosa* factors specifically affecting keratinocyte innate defense gene expression and on the identification of further keratinocyte genes specifically affected in their expression by these factors.

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Immunoblot Analysis of Various Protein Fractions of *Borrelia burgdorferi*, Including Variable Major Protein – Like Sequence Expressed during the Course of Erythema migrans Disease

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To examine the immune response against different proteins of *Borrelia burgdorferi* (*Bb*) in erythema migrans (EM) patients before and after therapy by an immunoblot test (IB) including the Variable major protein-like sequence, expressed (VlsE).

In 50 adult EM patients, *Bb* IgG and IgM antibodies were analyzed in a median of 5 serum samples consecutively obtained before and during a median of 513d (range, 414–1185d) after therapy by recombinant IB. The antigens used in the IgG IB were Outer surface protein (Osp) 17, p41i, OspC, VlsE, p39, p58, p100. In the IgM IB p41i, OspC, p39, and p100 were used. All serum samples were also tested by a standard IgG and IgM ELISA.

The IgG IB gave positive results in 50% before and in 57% directly after therapy (ELISA: IgG 22% and 25%, respectively). The respective values for IgM testing were: IB 36% and 43%; ELISA 42% and 61%. By the end of the follow-up period, IgG and IgM IB gave positive results in 44% and 12%, respectively. In acute and convalescence phase sera, VlsE was the most sensitive protein in the IgG IB (60% and 70% positive, respectively). In the IgM IB, p41i (46% and 57%) and OspC (40% and 55%) were the most sensitive proteins. By the end of follow-up, only the IgM response to p41i was significantly decreased to 24% (vs. 46% before therapy) ($p = .036$). The immune response to all other proteins did not change significantly over the follow-up.

The IgG IB was more sensitive than the IgG ELISA, whereas the IgM IB was less sensitive than the IgM ELISA. VlsE in the IgG IB and p41i and OspC in the IgM IB were the most sensitive proteins in acute and convalescence phase sera. During long-term follow up, only the immune response to p41i decreased significantly. Thus, the immune response against different *Bb* antigens is not influenced by antibiotic therapy.

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Expression of Toll-like Receptors in Normal Human Skin and Virus-Infected Skin Lesions

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The surface epithelium of skin plays a key role as the first line of defense against pathogenic microorganisms. Recent studies suggest that some of toll-like receptors (TLRs) are functionally expressed in normal human keratinocytes *in vitro*, although there have been controversies. However, little is known about the expression of TLRs in normal human skin *in vivo* and common virus infected skin lesions. In this study RT-PCR analysis was used to examine the mRNA expression of 5 TLRs (TLR2, TLR3, TLR4, TLR7, TLR9) in the normal human skin and the lesions of verruca vulgaris and molluscum contagiosum. Immunohistochemical staining was also performed to examine the pattern of TLR expression in normal human skin and viral lesions. Homogenized normal human skin constitutionally express TLR2, TLR4, TLR7, but not TLR3, TLR9. TLR7 was also expressed in the homogenized samples from verruca vulgaris and molluscum contagiosum. Immunohistochemical staining showed the strong expression of TLR2, TLR4, and TLR7 in whole epidermis of normal human skin. TLR3 and TLR9 were weakly expressed in the basal layer of normal human skin. TLR3 expression was diffusely augmented in the hyperkeratotic epidermis of verruca vulgaris and in the vicinity of molluscum bodies. There was little difference in the expression of other TLRs between normal human epidermis and viral lesions. These findings suggest that some TLRs can be implicated in the pathogenesis of skin viral infections such as verruca vulgaris and molluscum contagiosum.

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***Proprionibacterium acnes* Surface Proteins are Recognized by Extracellular Matrix Proteins and Keratin**

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Acne is a multifactorial disease of the skin, particularly the pilosebaceous unit. Acne lesions can be divided into noninflammatory and inflammatory lesions where the inflammatory lesions consit of papules, pustules and nodules where the follicular epithelium is damaged with a dermal inflammation. One of the factors promoting the development of inflammatory acne is the bacterial colonization of the pilosebaceus duct by the anaerobic *Proprionibacterium acne* (*P. acnes*) strain. In this study, we investigated the capability of *P. acnes* surface proteins to be recognized by extracelular proteins (collagens, fibrinogen) and keratin which is the major protein constituant produced in the keratinocytes. Two *P. acnes* strains, isolated from acne lesions, were used and grown on liquid and solid reinforced clostridium medias (RCM) containing or not Tween 80 and on solid blood agar. *P. acnes* surface proteins were heat extracted in presence of detergent and with LiCl, and then electrophoretically separated and tested for their capabilities to be recognized by biotinylated purified collagen I, IV, VI, VII ; fibrinogen and keratin. Overlay analysis of surface proteins extracted from bacteria grown on solid RCM showed, a band ranging from 62 to 70 kDa depending of the bacterial strain, recognized strongly by keratin, fibrinogen and weakly by collagen VI while Collagen I, IV and VIII were not. When comparing the extraction methods, only the LiCl based-extraction gave better recovery. These results indicate that *P. acnes* strain possess surface proteins able to be recognized by proteins synthetized in the keratinocytes. Proteomic characterizations of these surface proteins are underway.

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Characterization and Typing of *Trichophyton raubitschekii* Isolates. First Report from the South-East Region of Europe

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Trichophyton raubitschekii is classically isolated from African, South East Asian and Australian aboriginal patients with tinea corporis or tinea cruris. It is a debated dermatophyte species, differentiated from *Trichophyton rubrum* principally by its positive urease activity and production of profuse macroconidia and microconidia in culture. This study was undertaken to screen Greek and Bulgarian clinical isolates characterized as *T. rubrum* for *T. raubitschekii* and to delineate these strains by two molecular methods used for the first time in *T. rubrum* epidemiological studies.

Ninety-five Greek strains and 10 Bulgarian strains, originating from various body sites, initially identified as *Trichophyton rubrum,* were screened for urease activity in Christensen's urea agar. Subsequently, all urease positive strains were tested for lack of bacterial contamination, histidine growth independence and lack of *in vitro* hair perforation as well as for NaCl intolerance. Their morphology was studied in Sabouraud dextrose agar, potato dextrose agar and by slide cultures. Strains were delineated with PCR-ribotyping amplifying repeat elements of the Intergenic Spacer region (IGS) and by PCR fingerprinting utilizing a decameric minisatellite oligonucleotide sequence.

In total, five Greek and one Bulgarian *T. raubitschekii* strains were identified comprising isolates from patients with tinea manuum (1), tinea corporis (1), tinea cruris (1) and onychomycoses (3). Although all strains produced macroconidia, only one strain had the classical *T. raubitschekii* microscopic morphology, the other 5 presented a dominant arthroconidial phenotype. Only 3/6 *T. raubitschekii* strains originated from tinea corporis cases, whereas the majority of *T. rubrum* were onychomycosis isolates. Both typing methods, though not used before in dermatophyte typing, clustered all *T. raubitschekii* and *T. rubrum* isolates in the same group indicating strain homogeneity.

The surprising large autochthonous incidence of *T. raubitschekii* in the Balkan and in S.E. Mediterranean regions extends the geographical distribution of this species. This finding also highlights the importance of accurately recording the anatomical site of lesions caused by *Trichophyton* isolates, so as to perform pertinent differential biochemical tests for the reliable characterization of isolates. Although *T. rubrum* and *T. raubitschekii* infections are regarded as relatively known clinical entities, still a lot remains to be elucidated on their epidemiology and their respective response to treatment through phenotypic and genetic studies.

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Isolation of Quinolone-Resistant Ureaplasma Urealyticum and Identification of Gene Mutaions in Quinolone Resistant Determining Regions

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Objectives: To isolate quinolone-resistant UU (Ureaplasma Urealyticum) from clinical isolates and further to determine the gene mutations in QRDRs (quinolone-resistant determining regions) in such resistant isolates.

Methods. Two kinds of drug susceptibility test kits were used to determine the quinolone-resistant UU isolates from 44 clinical isolates. Those isolates with color changes from yellow to pink or red in both media with and without quinolones were considered as the resistant ones. Broth dilution method was used to dilute four kinds of quinolones with a series of concentrations from 0.125μg/ml to 128μg/ml in 96 well plates. The MIC was used to evaluate UU_3 reference strain and clinical isolates. The isolates with MICs four times higher than those of UU_3 for the same drugs were considered as resistant isolates. Two pairs of specific primers were used to amplify gyrA and parC, two major genes in QRDRs of UU. PCR was performed and the expected fragments for gyrA parC should be 336bp and 309bp respectively. The positive amplicons were sequenced and the results were compared with the reference strains.

Results. Of 34 tested isolates, 10 were resistant to both OFX (ofloxacin) and SPX (sparfloxacin), 15 only resistant to OFX and 1 resistant to SPX. Eight isolates were sensitive to both drugs. Of another 10 tested isolates, 6 were found resistant to CFX (ciprofloxin), and 3 resistant to SPX. For 4 kinds of quinolones tested, MICs of UU_3 reference strain were 1μg/ml for LFX (Levofloxacin), 4íg/ml for CFX, 8μg/ml for NFX and 2μg/ml for SPX. MICs of 2 out of 22 UU isolates were found four times higher than those of the reference strain. The amplified fragments with 336bp (gyrA) and 309bp (parC) were observed in 3 out of 6 resistant strains, 1 with positive parC band. Compared with the reference strain, the sequenced results revealed a C to A change at 87nt of gyrA in 1 isolate , a C to T change at 50nt of parC in 1 isolate and both changes in 1 isolate.

Conculsions. The results of drug susceptibility tests are important for clinical therapy in UU infection because of drug-resistant isolates. Drug susceptibility kits are easier and more convenient to apply than broth dilution method for further and wider usage. It is urgent to standardize the NGU therapy in the light of the susceptibility results. It can be implied that resistance of UU to quinolones may have some relation to the gene mutations in QRDRs such as gyrA and/or parC.

429 [Oral 019]

Adjuvant IFNα Therapy Stimulates Transporter Proteins Associated with Antigen Processing and Proteasome Activator 28 in Patients with Malignant Melanoma

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The use of cytokines, especially of interferon alfa (IFNα) for the treatment of metastatic melanoma was evaluated in several clinical trials. Although IFNα shows a broad spectrum of immunmodulatory and antiproliferative effects in a variety of malignancies the mechanisms of its antitumor effect and its action in adjuvant melanoma therapy remained unclear. In this clinical study we showed that expression of transport proteins associated with antigen processing (TAP1 and TAP2) and proteasome activator 28 (11S REG) was significantly upregulated by i.v. adjuvant treatment with 10 million IU/m^2 IFNα in 13 patients with malignant melanoma (stage III, UICC). This strong stimulatory effect was seen in blood mononuclear cells (PBMC) both on the RNA level using RT-, Real time- PCR and on the protein level using immunohistochemistry and immunoblotting. Depending on the patient analyzed, 2- to 5-fold upregulation of TAP1 mRNA expression could be detected by Real time- PCR. The finding that IFNα stimulates the cytotoxic effector functions in PBMC of patients receiving intermediate high dose immunotherapy by enhancing TAP expression and proteasome activity contributes to the understanding of the immunoregulatory role of type 1 interferons and may help to explain the efficacy of IFNα in the treatment of tumors. These parameters will also provide a new clinical tool for measuring the efficiency of INFα therapy *in vivo* and will give further information about the most effective dose and the duration of IFNα administration.

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Anti-Inflammatory Activities of the p38 MAP Kinase Inhibitor EO1606 in Dermatological Disease Models

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The amino-benzophenone EO1606 is a new, potent and selective inhibitor of p38 MAP kinase (isoforms α and β) and of the upstream kinase MKK6. *In vitro*, EO1606 inhibits the production of inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α from human mononuclear leukocytes. The anti-inflammatory effect of EO1606 was tested and characterized in the following dermatological disease models *in vivo*: In the acute oxazolone induced contact hypersensitivity and TPA-induced chronic irritant contact eczema models, the anti-inflammatory effect was comparable to that of group II/III corticosteroids. In the passive cutaneous anaphylaxis model, the effect was comparable to levocabastine hydrochloride. In the UV-B induced erythema model, the anti-inflammatory effect was by far more potent than ketoprofen. In the magnesium deficient induced dermatitis model, the effect was comparable to that of FK506. In the superantigen induced dermatitis model, no anti-inflammatory effect was observed. In summary, EO1606 shows a broad anti-inflammatory effect *in vivo* which may be beneficial in a number of human dermatological diseases.

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Dissociation of Transactivation from Transrepression Activity by a Selective Glucocorticoid Receptor Agonist (SEGRA) Leads to Separation of Therapeutic Effects From Side Effects

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Glucocorticoids (GCs) are the most commonly used anti-inflammatory and immunosuppressive drugs. Their outstanding therapeutic effects, however, are often accompanied by severe and sometimes irreversible side effects. Thus, the goal of GC pharmacological research is the development of new drugs which show a reduced side effect profile while maintaining the anti-inflammatory and immunosuppressive properties of classical GCs. GCs affect gene expression either by transactivation or transrepression mechanisms. The anti-inflammatory effects are mediated to a major extent via transrepression while many side effects are induced by a transactivation mechanism. Therefore, we aimed to identify ligands of the glucocorticoid receptor (GR) that preferentially induce transrepression while avoiding or at least strongly reducing transactivation. Here we describe a selected non-steroidal selective GR-agonist (SEGRA), ZK 216348, which shows a significant dissociation of transrepression and transactivation activities both *in vitro* and *in vivo*. In a murine model of skin inflammation ZK 216348 is anti-inflammatorily active comparable to prednisolone after both systemic and topical application. A remarkable superior side effect profile was found with regard to blood glucose induction, spleen involution and to a lesser extend skin atrophy but not to ACTH suppression. Accordingly ZK 216348 should have a lower risk e.g. for induction of diabetes mellitus. Thus, the SEGRAs represent a promising new class of drug candidates with an improved effect/side effect profile in comparison to classical GCs. Moreover, they are attractive tool compounds for further investigating the mechanisms of GR-action.

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Ultraviolet Radiation and Bradykinin Induce Erythema in Man Independently Through a Nitric Oxide-Dependent Pathway

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Bradykinin (BK) is a small peptide hormone released at sites of inflammation, and is an inducer of cutaneous erythema. Tissue culture and animal work has suggested BK-induced erythema may utilise nitric oxide (NO) as the final common pathway, but there are no human studies to substantiate this. Ultraviolet radiation (UVR) is also thought to induce erythema through production of NO, and in animal work, BK. We investigated the effect of the NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME) and the constitutive BK antagonist HOE 140 on BK and UVR-induced erythema in human volunteers.

Increasing concentrations of BK alone, or BK with L-NAME were infused into human forearm skin using cutaneous microdialysis catheters. UVR-exposed skin (UVB, xenon arc lamp, Lotoriel, UK) was infused with L-NAME or HOE-140. Erythemal response was measured as a function of cutaneous blood flow using laser doppler flowmetry (Moor Instruments, UK).

BK infusion resulted in cutaneous erythema at concentrations as low as 10 μM, and demonstrated an erythemal effect in a dose-dependent manner. BK-induced erythema was suppressed by the BK antagonist HOE-140. L-NAME inhibited the effect of BK-induced erythema at concentrations of BK up to 100 μM. This effect was pronounced, with erythema typically returning to near baseline levels. Infusion of the inactive isomer D-NAME with BK had no such effect. Thus inhibition of nitric oxide synthase abolished the ability of BK to induce cutaneous erythema. UVR-mediated erythema was inhibited by L-NAME infusion, but not by HOE-140.

The mechanism through which BK mediates erythema has been the subject of speculation. This is the first study in humans to demonstrate BK acting through a NO-dependent pathway, and that this occurs in a dose-dependent fashion. BK may induce NO production directly, or through a downstream product such as prostaglandin. Furthermore, we have shown that UVR-induced erythema acts independently of BK, but still through the NO pathway.

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Stimulation of Purinoceptors Induces the Release of Cytokines from Human Epidermal Keratinocytes

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In response to cell damage by mechanical stimulation, ATP is released from keratinocytes. We examined the effect of extracellular nucleotides on the induction and release of cytokines in cultured normal human epidermal keratinocytes (NHEK). Extracellular nucleotides increased the release of cytokines (IL-6 and IL-8) in a concentration-dependent manner. The order of potency for IL-6 release was ADP > ATP > 2-methylthio ADP ≫ UDP. Release of IL-6 was not induced by UTP or α, β-methylene ATP. The order of potency for IL-8 release was ATP = UDP = 2-methylthio ADP > or = ADP ≫ UTP. Release of IL-8 was not induced by α, β -methylene ATP. These effects of ATP (0.3 mM) were inhibited by P2 receptor antagonist suramin (0.1 mM) and P2Y receptor antagonist reactive blue 2 (0.03 and 0.1 mM), but not by P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2', 4'-disulphonate (PPADS). ATP also increased the expression of IL-6 and IL-8 mRNA using quantitative RT-PCR methods. These expressions were inhibited by suramin and reactive blue 2, but not by PPADS. Though release of IL-6 and IL-8 was increased in NHEK after UVB irradiation, these increases were also inhibited by suramin and reactive blue 2 and further, the augmentative expression of IL-6 mRNA by UVB was inhibited by suramin and reactive blue 2. Additionally UVB irradiation evoked the release of ATP from NHEK. In this study, we demonstrated that extracellular ATP could specifically stimulate expression and release of IL-6 and IL-8 by mediating through P2Y receptors in NHEK. These results indicated that there is a possibility that controlling purinoceptors may inhibit skin inflammation during disease processes.

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T-Cell Proliferation in Human Peripheral Blood Mononuclear Cells: Synergistic Inhibition by Combinations of Pimecrolimus with Corticosteroids

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Corticosteroids have been the mainstay of therapy for inflammatory skin diseases, (e.g., atopic dermatitis and psoriasis), as well as other chronic inflammatory conditions, where T-cell activation plays a key role. However, corticosteroid resistance has been reported frequently. Pimecrolimus (ASM) is an ascomycin macrolactam derivative specifically designed and developed to treat inflammatory skin diseases. Some *in vitro* T-cell systems (e.g., stimulated with superantigen and anti-CD3/CD28) have been documented to exhibit resistance to corticosteroids and/or calcineurin inhibitors. In this study we examined the effects of corticosteroids and pimecrolimus on T-cell proliferation in human peripheral blood mononuclear cells (PBMC) stimulated with the superantigen staphylococcal enterotoxin B (SEB) or with the combination of anti-CD3 plus anti-CD28 mAb for 72 hr. Proliferation was measured via incorporation of 5-bromo-2'-deoxyuridine (BrdU).

When used as single agents in the present study, the corticosteroids dexamethasone (Dex) and betamethasone 17-valerate (Beta) at 300 nM and hydrocortisone (HC) at 10,000 nM, as well as pimecrolimus at 30 nM exerted, at most, only partial inhibitory effects on T-cell proliferation. However, combinations of the corticosteroids with pimecrolimus (at the concentrations indicated above) exhibited strong and synergistic inhibition of proliferation. The following ranges of inhibition (stimulated control = 0%) were observed: SEB-induced proliferation: corticosteroid (⩽15%), ASM (4–33%), corticosteroid plus ASM (⩽93%). Anti-CD3 + anti-CD28-induced proliferation: corticosteroid (⩽25%), ASM (⩽21%), corticosteroid plus ASM (61–95%). These results suggest that combination therapy may be efficacious to overcome corticosteroid resistance and/or for indications, where corticosteroid or pimecrolimus monotherapy is insufficient.

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Transungual Delivery of Drugs: New Perspectives

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Antimycotic nail lacquers show a rather low rate of clinical successes in patients with onychomycosis, despite their proven *in vitro* activity. The formulations available till now have the disadvantage of poor nail permeation of the active principles, as well as need of weekly removal of the old layers. Both mechanical removal and organic solvents, besides being uncomfortable to patients, may cause a potential damage to the nail structure, by rendering the newly growing nail less resistant to the diffusion of the dermatophytes. The use of nail penetration enhancers, besides improving the nail permeation, may further damage the nail structure as a result of dysulphur bond breaking.

A new proprietary technology of "transungual" water solutions has been developed, acting as a carrier at the nail level, by employing chitin derived hydrosoluble amino-polysaccharides. The new technology is based on hydroalcoholic solutions of hydroxypropyl chitosan (HPCH), a water soluble semi-synthetic derivative of chitosan, which acts as a film forming agent. HPCH dissolves in high percentage in water, has affinity to air, is a highly plastic substance and forms a highly elastic film, it increases the dispersion of other ingredients, it is an excellent carrier, finally it has neither toxicity nor irritation. An 8% ciclopirox solution in the new formulation vehicle was able to permeate 150 μm bovine hoof membranes as *in vitro* model of human nails, with a lag time of 3.36 ± 0.46 hours and a flux of 4.70 ± 0.60 μg/cm^2.h, compared to a lag time of 12.48 ± 1.31 hours and a flux of 3.05 ± 0.63 μg/cm^2.h of a reference solution containing a reference solution of a traditional polyvinyl resin as a film forming agent. After 30 hours from a single application of the new formulation, 11% of the applied ciclopirox amount was already penetrated into the nail membranes. In our experience, HPCH alone, unlike other chitosans, did not show any antimycotic property, but it potentiated the inhibiting activity of several antimycotic agents on the *in vitro* growth of *T. mentagrophytes*. *In vitro* studies on MICs of new ciclopirox formulation showed MIC ⩽0.0015% for *T.rubrum*, ⩽0.0015% for *C. parapsilosis* and = 0.2% for *S. brevicaulis*, as ciclopirox percentage. *In vitro* experimental infections on bovine hooves showed an excellent activity of the new ciclopirox formulation both in the preventive and in the curative test (100% of both) in experimental infections by *T. menta-grophytes*, by *T. rubrum* as well as by *M. canis*. An investigation in healthy volunteers showed that a 30% of the applied dose of CPX was detected in fingernails washed by water and soap already 6 hours after a single application of ciclopirox and HPCH solution 10 μl. The ciclopirox concentrations found in those subjects after washing were in the order of magnitude of μg/mg, far above ($>10^3$) the above MICs.

Chitosan and its derivatives possess adhesive properties towards different biological tissues due to their positive charge. Moreover, the free hydroxypropyl groups of HPCH may interact with keratin, by hydrogen bonding and other weak interactions that could contribute to the improved drug transport and release. The nail application of the new formulation, even for chronic treatments like in onychomycosis, is easy and accepted by the patients due to the simple (rinsing) removal procedure and no need of nail filing.

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EO1606 – a p38 MAP Kinase Inhibitor as a Potential Treatment of Acne

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The amino-benzophenone EO1606 is a new, potent and selective inhibitor of p38 MAP kinase (isoforms α and β) and of the upstream kinase MKK6. The compound has a broad anti-inflammatory activity, inhibiting the production of a number of inflammatory cytokines including interleukin 1β and tumour necrosis factor-α. We tested this compound in an animal model of non-inflammatory acne, the rhino mouse. Dermal application of EO1606 decreased the number of pseudo comedones and induced a normalisation of the follicular unit in the skin. The changes were associated with epidermal hyperplasia. The efficacy of EO1606 in the rhino mouse is comparable to that of the retinoids, which are established as very efficacious treatments of acne. Immunohistochemical staining of rhino mouse skin showed phospho-p38 staining of single scattered cells in the basal layer of the epidermis. No staining was seen in EO1606-treated skin.

Acne vulgaris is a multifactorial disease characterised by increased sebum production, comedone formation, infection with *P. Acnes* and inflammation.

EO1606 targets two of these factors, inflammation and comedone formation, and its efficacy for acne vulgaris was tested in a clinical trial. However, EO1606 did not prove efficacious in this trial.

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Efalizumab – A Targeted Modulator of T-Cell Activity for the Treatment of Plaque Psoriasis

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Psoriasis is a chronic, immune-mediated skin disease affecting as many as 1 in 50 people. It is characterized by thickened, erythematous plaques covered in silvery scales that arise from excessive proliferation and abnormal differentiation of keratinocytes in the epidermis. Underlying the pathogenesis of psoriasis is an abnormal, sustained immune response mediated primarily via CD8 + T cells. The antigen(s) responsible for activating the T cells has yet to be identified. Standard systemic therapies for treating moderate to severe psoriasis all suppress T-cell activity, but they also all have serious adverse effects on other cells in the body that limit their long-term use. Efalizumab is a recombinant humanised IgG_1 monoclonal antibody targeted against the CD11a site of leukocyte function–associated antigen (LFA)-1, the predominant integrin expressed on T cells. LFA-1 normally binds to intercellular adhesion molecule (ICAM)-1 expressed on antigen-presenting cells (APC), endothelium, and keratinocytes. LFA-1 is a key element in the mature immunological synapse, forming a ring of adhesion molecules (the P-SMAC) around a stable central cluster of T-cell receptors (the C-SMAC). Disruption of LFA-1/ICAM-1 binding by efalizumab inhibits multiple T-cell functions associated with the pathogenesis of psoriasis, including activation of T cells by APC, the trafficking of T cells to the dermis and epidermis, and interaction of T cells with keratinocytes. Blockade of LFA-1 may also prevent the cytotoxic activity of fully differentiated CD8 + T cells on keratinocytes in lesional epidermis. In patients, efalizumab rapidly saturates and downregulates expression of CD11a sites within 24–48 hours after a subcutaneous (SC) dose. Weekly SC doses of efalizumab 1 mg/kg completely downmodulates CD11a on T cells, effectively preventing T-cell activation and trafficking and reversing the histological skin changes seen in psoriatic lesions. Because efalizumab is T-cell sparing, it potentially offers a safer alternative to currently available therapies and requires less monitoring in clinical practice.

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***tetra*-Isopalmitoyl Ascorbic Acid as an Anti-Aging Agent**

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Deep/fine wrinkles and pigmented spots characterize aging of facial skin. Environmental UV light largely contributes to the acceleration of skin aging. Reactive oxygen species (ROS) are proposed as one of mechanisms of UV-accelerated skin aging (also called photoaging of skin) from various investigations. From the evidence, antioxidants such as ascorbic acid (AsA), dl-α-tocopherol, glutathione and cysteine derivatives are widely used to prevent the formation of photoaging skin. It is well known that AsA in antioxidants is a multi-functional vitamin on skin homeostasis. Certainly, the antioxidative property of AsA exhibits the protection of skin from various oxidative stresses such as UV irradiation and reactive oxygen species (ROS). AsA has also been used as a typical skin lightning agent in cosmetic field due to its inhibitory effect against a key enzyme of melanin formation, tyrosinase. In addition, AsA, which plays a critical role in dermal construction, stimulates collagen synthesis of dermal fibroblasts. In recent study, it was documented that AsA regulated epidermal differentiation due to regulation of differentiation-relating genes. Although AsA exhibits these effects on skin homeostasis, there are still some obstacles to wide application of AsA: e.g. AsA hardly penetrates to the target cells through the skin and has lower stability in formulations. In the point of stability, phosphoryl ascorbate magnesium salt (VCPMg) has been developed and widely used, although the problem of penetration from skin surface still remains. Then, we synthesized *tetra*-Isopalmitoyl ascorbic acid (VC-IP) to minimize the disadvantages of AsA. In this presentation, we will discuss the potential of VC-IP as an anti-aging agent, focusing on its anti-oxidative effect.

At first, we examined whether VC-IP exhibited reduction effect on various oxidative stress using cultured HaCaT cells. In general, UVB generates ROS in cells through several pathways, and consequently UVB causes oxidative stress into cells. As well established, ROS injure DNA, lipids and proteins in the cells, and ultimately lead to cell death. Therefore, we checked the reduction of DNA damage induced by UVB irradiation using Comet assay. VC-IP significantly suppressed the elongation of comet tail suggesting the reduction of the DNA damage. In addition, VC-IP showed the decrease of cell death after UVB exposure. ROS generate peroxides in the cells by attacking lipids and proteins. It is well established that UVB-irradiated cells show the higher peroxide level. The treatment of cells by VC-IP before UVB irradiation significantly reduced intracellular peroxide level. In addition, it was found that VC-IP reduced other oxidative stress initiated by exposure of ROS, superoxide anion, hydrogen peroxide and singlet oxygen. From these results, we can expect VC-IP to act as an effective anti-aging agent.

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Anti-Aging Effects of d-δ-Tocopheryl Retinoate – Evaluation Using Cultured Skin Cells

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Skin aging, which is characterised by wrinkles and sagging is caused by structural alterations of dermis, decrease of collagen and denaturation of elastin. It is well established that these alterations are accelerated by UV light. All-*trans*-retinoic acid (RA) is widely prescribed for treatments of various diseases, and also used for treatment of photoaged skin. However, since RA expresses severe skin irritation, its general application in drugs and cosmetics is difficult. To improve this disadvantage, we synthesised a new RA-derivative, d-δ-tocopheryl retinoate (TR), and evaluated its potential as anti-aging agent focusing on collagen condition.

First of all, to examine whether TR exhibits same effect as RA, we evaluated hyaluronan (HA) synthesis in keratinocytes. TR showed an effective elevation of the hyaluronan synthesis at both HA synthase-3 mRNA level and HA amount. The result indicated that TR possesses the potential of RA.

In the skin exposed to UVB, ROS generation through few pathways has been established. ROS stimulate several cell functions, which lead to alterations of dermal collagen statue. First of all, UVB damages DNA by photo-chemical reaction and ROS. The effects of TR on DNA damage of HaCaT cells was examined by comet assay. TR suppressed the elongation of comet tail, suggesting reduction of UVB-induced DNA damage. It is well known that dermal collagen statue is regulated by a balance of synthesis and digestion. TR suppressed expressions of both matrix metalloproteinase (MMP)-2 and MMP-9 of HaCaT cells following UVA irradiation. In addition, TR elevated type I collagen synthesis of human dermal fibroblasts.

Recently, it has been demonstrated that the decrease of collagen in aged skin is caused by excessive expression of MMPs and decrease of collagen synthesis. Therefore, our test results prove that TR works as a new anti-aging agent through regulating the balance of collagen synthesis and digestion and can be utilized as a substitute of RA.

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The Human Haarscheibe Revisited

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The Haarscheibe, also referred to as touch dome is a structure within hairy skin considered to be a slowly adapting mechanoreceptor. Whereas it has been well characterized in hairy skin of cats and rats, only few studies address its presence in human skin. Using dermal sheet preparations we were able for the first time to identify and analyse entire Haarscheiben in human hairy skin *in situ* by confocal laser scanning microscopy. They appear as dense convolutes of the subepidermal nerve plexus and are present in the papillary dermis at a frequency of 1 to 2 per cm^2. Corresponding epidermal sheets show dense aggregations of Merkel cells with a mean number of ∼ 140 Merkel cells per Haarscheibe, embedded in a characteristic epidermal architecture. Surprisingly both myelinated and unmyelinated fibers supply the Haarscheibe. At least 2 myelinated fibers enter each receptor structure and the myelin sheath extends up to 10 μm below the dermo-epidermal junction. Each myelin sheath contains a neurofilament positive fiber, which after loss of its myelin sheath further divides into smaller fibres. In contrast to the myelinated fibers the unmyelinated ones are present as both neurofilament positive and negative. In conclusion, we were able to demonstrate for the first time the anatomical structure of the human Haarscheibe in its entirety. Our additional data on the diversity of nerve fibers present within the human Haarscheibe suggest that this unique nervous structure has the potential to serve as receptor for other sensorial qualities in addition to it attributed function as mechanical sensor.

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Gap Junctional Intercellular Communication and Cx43 Expression in Human Melanocytes and Melanocytic Lesions

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During development, melanocytes migrate from the embryonic neural crest into the basal layer of human epidermis where they synthesise melanin for transfer, via melanosomes, to keratinocytes. In the epidermis, melanocytes and keratinocytes form heterocellular associations primarily based upon E-Cadherin. These are disrupted during nevus formation and in the development of malignant melanoma, in favour of melanocyte-melanocyte or melanocyte-fibroblast interactions. Keratinocytes are connected throughout human epidermis via gap junction channels that allow intercellular communication between neighbouring cells. Within the epidermis, keratinocytes have a regulatory function over melanocytes in the basal layer, helping maintain the integrity of the Epidermal Melanin Unit. However, it is unclear whether or not melanocytes, nevocytes or melanoma cells are integrated within the gap junction network of human skin.

In this study, Cx43 expression in melanocytes was evaluated in normal epidermis, various types of melanocytic lesions, progressive stages of malignant melanoma and also by employing differing culture conditions.

Using double immunofluorescence analysis of Cx43 – in conjunction with melanocytic markers – melanocyte-keratinocyte cocultures, melanocyte monocultures and excised paraffin embedded melanocytic lesion materials, were evaluated for Cx43 gap junction expression. Potential cell-cell communication between melanocytes in monoculture and coculture with keratinocytes was determined via the intracellular iontophoresis of Lucifer Yellow and Alexafluor 350. Additionally, also employing dye transfer assays, the study investigated whether the application of forskolin, an agent known to increase cAMP levels and promote gap junctional communication in some cell lines, had any effect upon melanocyte cell-cell communication.

The results showed that in normal epidermis, Cx43 did not co-localise to melanocytes in the basal layer, to nevocytes in the majority of benign nevi or to melanoma cells in progressive malignant melanoma lesions, but, Cx43 was expressed in the deeper regions of compound nevi where it co-localised with many nevocytes. Indeed, Cx43 co-localisation was also observed in melanocyte monocultures, where a minority of cells showed perinuclear intracellular stores of Cx43. Moreover, dye transfer studies involving the application of forskolin to such cultures, demonstrated that both Lucifer Yellow and Alexafluor 350 were successfully transferred between a minority population of melanocytes in monoculture. However, in coculture with keratinocytes there was no successful dye transfer.

Under normal conditions, melanocytes do not appear to be integrated within the epidermal gap junction network. However, this study reports that in conditions when keratinocytes are absent or unable to exert a regulatory effect, in some instances, melanocytes can form functional Cx43 incorporated gap junctional communication channels with one another, both *in vitro* and *in vivo*.

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Is There a Potential Role for Dermal Mast Cells in the Hyperpigmentary Mechanism of Café-au-Lait Maculae in Neurofibromatosis Type 1 Patients?

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The etiopathogenesis of hyperpigmentary café-au-lait macules (CALMs) in Neurofibromatosis type 1 (NF1) patients is still very obscure. It has been suggested that increased secretion of cytokines (SCF/HGF) by dermal fibroblasts might be associated with accentuated epidermal hyperpigmentation in NF1 CALMs. Whether increased melanogenesis and/or melanocytic proliferation in NF1 CALMs is directly influenced by these fibroblast-derived cytokines or whether other cells (mast cells) could function as intermediate actors, is still unknown. In trying to answer this last question, we set up a quantitative study of the epidermal melanocyte and dermal mast cell population. Two parameters (with two values each) were included: genotype (non-NF1/NF1) and 'skin type' (normal/CALM). This enabled us to generate four groups: non-NF1 normal skin, non-NF1 CALM skin, NF1 normal skin, NF1 CALM skin. Immunohistochemistry was performed on paraffin embedded skin sections, using melan-A and c-kit antibodies to visualize melanocytes and mast cells, respectively. In-house counting and measuring software was developed and a standardized work flow was set up to accurately determine the number of melanocytes per mm basal membrane and mast cells per mm^2 dermis. This two-by-two factorial design was analyzed by a two-way ANOVA statistical approach splitting our main question in three: does genotype affect the number of cells? Do the number of cells change between skin types? Is genotype effect the same at the different 'skin types' (interaction)? We observed a main genotype effect on melanocyte number, showing a $\sim$1.5 to 2-fold increase in NF1 skin compared to non-NF1 skin (non-NF1 normal skin: 11.28 ± 0.82 ($n = 6$); non-NF1 CALM skin: 11.67 ± 1.59 ($n = 8$); NF1 normal skin: 17.82 ± 0.45 ($n = 3$); NF1 CALM skin: 19.34 ± 1.19 ($n = 6$)). However, the number of melanocytes did not change between skin type (no main skin type effect) and the observed increase due to genotype was independent of skin type, showing no significant difference between normal and CALM skin within each genotypic background (no interaction). There was also a genotypic effect on dermal mast cell number showing a $\sim$ 2-fold increase in NF1 skin (non-NF1 normal skin: 33.38 ± 7.34 ($n = 6$); non-NF1 CALM skin: 62.04 ± 3.81 ($n = 8$); NF1 normal skin: 69.84 ± 6.85 ($n = 3$); NF1 CALM skin: 64.11 ± 4.56 ($n = 3$)). However, the number of mast cells did not significantly change between skin type (no main skin type effect) but the observed increase due to a genotype effect was not the same on all skin type levels, showing a significant difference between normal and CALM skin within a non-NF1 genotypic background compared to NF1. The number of mast cells in NF1 normal skin was elevated compared to non-NF1 normal skin but the number of mast cells in CALM skin did not significantly differ between the two genotypes (interaction between genotype and skin type with main genotype effect). These observations may suggest that the mechanism of epidermal hyperpigmentation in CALMs, whether they are from a non-NF1 or NF1 patient, may be associated with an increase in dermal infiltrated mast cells (and associated cytokine secretion) and might be independent of NF1 heterozygosity.

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The Role of Fas System [Fas-FasL] as an Inducer of Apoptosis in Cutaneous Leishmaniasis: A Combined Human and Experimental Study

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Aim: To investigate the role of Fas system (Fas-FasL) as an inducer of apoptosis and other leishmanicidal cytokines in the disease development in cutaneous leishmaniasis.
Material and Method. Leishmanial skin and serum from both infected human and Swiss-albino mice were collected, Soluble Fas was estimated by ELISA, other leishmanicidal cytokines were detected by immunohistochemistry and tissue mRNA Fas by RT-PCR.
Results. Significant increase in interferon gamma, tumor necrosis factor-alpha, nitric oxide and soluble Fas in both infected human and mice. Tissue Fas showed marked expression in human samples while in human samples; the expression was reduced in chronic infected mice than in the late acute infected animals.
Conclusion. This study confirms the role of Fas expression in development of leishmanial lesion and show that Fas-Fas L pathway is a potent inducer of apoptosis which can help in complete resolution of cutaneous leishmaniasis. Therefore the future use of Fas-FasL as a novel therapeutic regimen may accelerate healing of leishmanial lesions.

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Clinical, Molecular and Immunohistological Assessment of Cutaneous Vasculitis in Chronic HCV Infection: Implications for Pathogenesis

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Introduction. Cutaneous vasculitis (CV) presents a diagnostic and therapeutic challenge. Hepatitis C virus (HCV) has been recognized in association with various forms of cutaneous vasculitic eruptions.
Aim. To detect the presence of HCV-RNA in skin of CV and to investigate its role in the pathogenesis of CV. Therefore, nuclear factor Kappa B (N-KappaB) and vascular endothelial growth factor expressions were evaluated. To clarify the role of infiltrating cells, endothelium and virus in CV with chronic HCV.
Patients & Methods. Classified into 3 groups .GpI included 27 HCV RNA positive patients with CV. GpII consisted of 11 HCV RNA negative patients with CV.GpIII 25 HCV RNA positive patients without vasculitis. Skin specimens were divided into 3 parts; one was kept frozen for N-KappaB. The second was fixed in formalin for immunohistology and PCR *in situ* hybridization. The third was prepared for electron microscopic study. The blood was subjected for circulating VEGF and N-KappaB.
Results. Revealed the presence of HCV-RNA in skin of 78% of GPI, none in GPII and 48% in GPIII. HCV particles were detected by EM in vascular endothelial and epithelial cells. There was significant expression of NF kappa B and circulating VEGF in Gp I, III then II respectively. The extent of histological changes is predictive of local clinical severity only but not correlated with the severity of liver damage.
Conclusion. HCV plays a major role in the pathogenesis and clinical recurrence of CV. HCV – RNA potentiates NF- kappa B in most cell types which in turn contribute to the chronically activated persistent stat of HCV infection in liver and frequent recurrence of CV. Therefore better understanding of these molecular events will provide a basis for rational design controlling frequent recurrence of CV and targeting HCV and its immunological sequels.

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Contractile Force and α-Smooth Muscle Actin Expression in Cultured Fibroblasts from Striae Distensae

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Striae distensae are characterized by linear, smooth bands of atrophic-appearing skin that are reddish at first and finally white. They are due to stretching of the skin, as in rapid weight gain, or mechanical stress, as in weight lifting. The pathogenesis of striae distensae is unknown but probably relates to changes in the fibroblast phenotype. In order to characterize striae distensae fibroblasts, α-smooth muscle actin expression (α-SM actin) and contractile forces were studied. Five healthy women with early erythematous striae on the thigh and five healthy women with older striae were selected. Paired biopsies were taken from the center of lesional striae and adjacent normal skin. Fibroblasts were obtained by an explant technique and expanded *in vitro* in Dulbecco's modified Eagle's medium. α-SM actin expression was studied by immunofluorescence labelling of cells cultured in monolayer. Contractile forces generated by fibroblasts in collagen latttices were measured with the Glasbox® device developed in our laboratory. Fibroblasts from early striae distensae were the richest cells in α-SM actin filaments and generated the highest contractile forces. Their peak contractile force was 26% greater than normal fibroblasts. In contrast, there was no significant difference in force generation between old striae fibroblasts and normal fibroblasts with cells expressing no α-SM actin. The contractile properties of fibroblasts from striae distensae varies depending on the stage of the disease. In early striae distensae, fibroblasts acquire a more contractile phenotype, corresponding to that of myofibroblasts.

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Contractile Properties of Cultured Human Fibroblasts from Chronic Venous Leg Ulcers

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The tissue contraction phenomenon associated with wound healing is of prime importance for wound closure. Contractile properties of human fibroblasts from chronic venous leg ulcers were compared to those of normal fibroblasts using *in vitro* models. Biopsies were taken from the uninvolved skin of the thigh, the epithelialized ulcer edge and the non-epithelialized ulcer center of 4 patients. Fibroblasts were obtained by an explant technique and expanded *in vitro* in Dulbecco's modified Eagle's medium. Alpha-smooth muscle actin expression (α-SM actin) was studied by immunofluorescence labelling of cells cultured in monolayer. Contractile forces generated by fibroblasts in collagen latttices were measured with the Glasbox® device developed in our laboratory. Fibroblasts from the ulcer centre were the richest cells in actin filaments. The peak contractile force developed by fibroblasts from the ulcer centre and the ulcer edge were respectively 30% and 18% greater than normal fibroblasts. Fibroblasts from venous ulcers have greater contractile capacity than normal fibroblasts. Fibroblasts from the ulcer center develop more stress fibers than fibroblasts from the ulcer edge; this could be responsible for the highest contractile force produced by fibroblasts from the ulcer center. The force of wound contraction is probably generated by actin filaments and transmitted to the sides of the wound. Contraction, which is a wound healing functional parameter, seems not to be affected in chronic venous ulcers.

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Expression of Cathepsin L and its Inhibitor Hurpin in Normal and Diseased Skin

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Hurpin (serpin B 13; PI13) is an intracellular, differentially spliced member of the serpin superfamily that has been linked to differentiation and apoptosis of human keratinocytes. It is transiently downregulated by UV light and overexpressed in psoriatic skin lesions. Recent reports indicate that hurpin is a potent and selective inhibitor of the lysosomal cysteine proteinase cathepsin L (Cat L). It is proposed that a physiological role of hurpin is to protect epithelial cells from ectopic Cat L.

Cat L, the papain-like lysosomal cysteine protease is known to be involved in apoptosis, tumor progression and metastasis, chronic inflammation and bone resorption. It is thought that Cat Ls ability to degrade extracellular matrix and basement membranes lets tumor cells to penetrate the interstitial tissue.

The expression of Cat L and hurpin in normal and diseased skin was investigated by immunohistochemistry and polymerase chain reaction (PCR) in order to better understand their pathophysiological role. Formalin-fixed and paraffin-embedded tissues from normal and psoriatic skin, lichen planus, verruca seborrhoica, actinic keratosis, squamous cell carcinoma, basal cell carcinoma and malignant melanoma were studied using an antibody against Cat L and newly generated monoclonal antibodies against hurpin. Additionally, expression was analysed by polymerase chain reaction (PCR) using RNA from normal and diseased skin biopsies.

We found that hurpin is overexpressed and redistributed in diseased compared to normal skin.

Whereas hurpin is mainly expressed in the basal layer in normal skin, we detected hurpin in the granular and squamous cell layer in diseased skin. Suprisingly, the distribution of Cat L did not correlate to that of hurpin. The highest expression of Cat L was found in the squamous cell layer with no profound differences between normal and diseased skin. For both proteins, we detected inter-individual differences in the expression level. Results from immunohistochemistry were confirmed by PCR data. Discordant expression of hurpin and Cat L suggests additional functions for this inhibitor. Inactivation of other intrinsic or extrinsic proteases might be important for the protective activity of the skin against infections.

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Diagnostic Applicability of *In Vivo* Confocal Laser Scanning Microscopy in Melanocytic Skin Tumors

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In vivo confocal laser scanning microscopy (CLSM) represents a novel imaging tool that allows examining skin morphology in real-time at a resolution equal to that of the conventional microscopes used to view histology slides. The imaging procedure, however, is non-invasive and does not require the use of fluorescence, dyes or stains.

The aim of the study was to test the applicability of CLSM to the diagnostic discrimination of benign common nevi (BN) and malignant melanoma (MM) by means of morphologic features.

In total, 117 melanocytic skin tumors (90 benign common nevi and 27 malignant melanomas) were imaged using a commercially available, near-infrared, reflectance confocal laser scanning microscope (VIVASCOPE 1000, Lucid Inc., NY, USA). At least 5 images comprising all epidermal layers and the papillary dermis were recorded in each case. 5 independent observer (2 residents, 1 senior physician and 2 dermatopathologists) without previous experience in CLSM received a standardized instruction about diagnostic CLSM features of nevi and melanoma. Diagnostic features comprised melanocyte cytology (round-oval, monomorphic in BN versus large and polymorphic cells in MM), architecture (regularly arranged nevus cell nests versus architectural disarray in MM), brightness of image (homogeneous brightness in BN versus irregularly scattered brightness in MM), dendrites (infrequent, simple branching in BN versus frequent, complex branching in MM) and keratinocyte cell borders (readily detected in BN versus poorly defined in MM). For diagnostic assessment, 2 diagnostic images in each case were shown to the observer on the computer screen and evaluated as belonging either to benign nevi or malignant melanoma. Additionally, presence or absence of each of the features were assessed by two of the observers. Interobserver reliability data were produced in the form of the kappa statistic for each feature.

Best performance was achieved by the two residents with a sensitivity value of 96.3% and specificity of 100 and 98.9%, respectively, followed by the senior physician without dermatopathologic qualification with 92.6 and 98.0%. Sensitivity of 96.3 and 56.3% and specificity of 93.3 and 95.6% was reached by the two dermatopathologists. When the presence or absence of morphologic features were assessed, it turned out that mainly architecture, cytology and keratinocyte cell border should be taken into account for diagnostic decisions, whereas image brightness and dendrites were rarely useful.

To our knowledge, this is the first study dealing with sensitivity and specificity in diagnostic assessment of CLSM images of melanocytic skin tumors. Obviously, a considerable high diagnostic performance could be achieved. The fact that observers without specific dermatopathology experience performed better than trained dermatopathologists indicates that micromorphologic features relevant in paraffin histology cannot directly be related to CLSM images. An unprejudiced approach of the observer seems to be more successful with respect to diagnostic accuracy.

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Cyclosporin A Induces Anagen and Increases the Number of Dermal Papilla Fibroblasts in Nude(Foxn1$^{nu/nu}$) Mice

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Phenotypically nude(Foxn1$^{nu/nu}$) mice have no thymus and show hair and nail abnormalities. Hair shafts bend and coil when they enter the hair canal, dilating the infundibulum and the hair shaft does not penetrate the epidermis. In nude mice, the immunosuppressant cyclosporin A (CsA), which causes hypertrichosis in man, induces macroscopically visible hair growth. In normal C57BL/6 mice, CsA induces anagen and inhibits catagen. The current study aimed at finding out whether CsA has any influence on hair follicle cycling and on dermal papilla cell number in the hair follicles of NMRI$^{Foxn1nu/nu}$ mice. Homozygous NMRI$^{Foxn1nu/nu}$ mice were treated with CsA (30 mg/kg/d i.p.) or vehicle (corn oil) for 14 days. Back skin was harvested, snap-frozen/paraffin-embedded and analysed for quantitative immunohistomorphometry. Cell proliferation and apoptosis were assessed by Ki67 and TUNEL immunostains. The number of DAPI-positive dermal papilla fibroblasts was counted in early anagen hair follicles of CsA-treated and control mice. CsA-induced anagen in telogen hair follicles of NMRI$^{Foxn1nu/nu}$ mice and partially normalized hair shaft structure, thus allowing it to penetrate the epidermis. No differences were noted in the rates of keratinocyte proliferation or apoptosis between CsA-treated and control NMRI$^{Foxn1nu/nu}$ mice. However, in early anagen hair follicles of CsA-treated NMRI$^{Foxn1nu/nu}$ mice, a significant increase in the number of DAPI-positive dermal papilla cells was visible compared to control mice, suggesting increased migration of fibroblasts from the connective tissue sheath (CTS) to the DP. These findings underscore the migratory activity of specialized hair follicle fibroblasts between CTS and DP (Tobin *et al*, JID120:895–904, 2003). Also they suggest that CsA may stimulate immigration of morphogenically active fibroblasts into the DP. Together with its anagen-inducing effects, this may underlie the hypertrichosis-inducing effects of CsA seen in clinical practise.

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LEKTI, Terminal Differentiation of Keratinocytes and Skin Barrier Formation

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LEKTI (LymphoEpithelial Kazal Type serine protease Inhibitor) is encoded by the SPINK5 gene, mutations of which caused Netherton's syndrome (NS) as a result of non-expression of functional LEKTI. Clinically, NS has similarities to atopic dermatitis (AD) which include chronic inflammation of the skin, multiple allergies and high IgE levels. Genetic polymorphisms in the SPINK5 gene have been demonstrated in AD. The biological role of LEKTI is currently unknown and it is hoped that a better understanding of its function may provide an insight into the pathogenesis of AD. Skin barrier function in NS is severely compromised. LEKTI is expressed in the stratum granulosum of normal skin, absent in NS and abnormal in AD. This places LEKTI at a strategic position to influence the skin barrier and given the clinical features of NS, we postulate that LEKTI may play a role in influencing terminal differentiation of keratinocytes and skin barrier formation. Using immunofluorecence techniques, we investigated the process of keratinisation in normal skin (n = 4), NS (n = 4) and AD (n = 3). Expression patterns of differentiation markers, such as involucrin, transglutaminase 1, loricrin, keratin 6 and 14 were studied. To further evaluate the biological role of LEKTI, cultured keratinocytes were stimulated with calcium and epidermal growth factor (EGF). The response of LEKTI to these stimuli was analysed by flow cytometry. Skin specimens from NS patients showed an abnormal expression of involucrin and loricrin. In normal skin, these proteins were expressed in the stratum corneum in a membranous pattern whereas in NS, they were expressed in the cytoplasm and found in most of the suprabasal layers. K6 expression was increased and K14 reduced when compared to normal controls. These findings suggest a disrupted process of keratinisation and terminal differentiation in NS. In AD, expression of K6 was increased similar to NS but all other proteins were normal. The flow cytometry data revealed that LEKTI level was increased when keratinocytes were stimulated with calcium and EGF which are well known modulators of keratinocytes differentiation. Up-regulation of LEKTI by these two factors suggests that LEKTI is involved in keratinocyte differentiation. These findings combined with the abnormal staining results in NS suggest that LEKTI may influence terminal differentiation through the calcium metabolism pathway for example via PAR-2 and subsequently modulate transglutiminase activity.

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Limitations of Dermatoscopy in the Diagnosis of Melanoma

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The aim of this study was to compare dermatoscopic features of melanocytic nevi with those of early melanomas, which were not excised initially because of their uncharacteristic clinical and dermatoscopic appearance. We included the dermatoscopic images of 325 melanocytic skin lesions, which were followed by digital dermatoscopy and finally excised because of changes over-time and compared the base-line images of 63 melanomas and 262 melanocytic nevi by pattern analysis, the ABCD rule, and the 7-point checklist. The patterns of dermatoscopic features observed in the base-line images of those melanocytic lesions, which were finally diagnosed as melanomas during follow-up, did not differ substantially from the patterns observed in the base-line images of melanocytic nevi. Pattern analysis, the ABCD rule, and the 7-point checklist failed to achieve a satisfying diagnostic accuracy for melanoma. In retrospect, no dermatoscopic feature or pattern of features could be identified, which reliably differentiated between melanomas and melanocytic nevi at the time of the first presentation of the patient. In conclusion dermatoscopy depends on the appearance of classic dermatosopic features and is therefore limited in the diagnosis of very early and mainly featureless melanomas.

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Alterations of Basement Membrane Zone in Lichen Planus Differ from that in Lichen Sclerosus

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The etiopathogenesis of lichen sclerosus (LS) is not clear but an association with various autoimmune diseases and lichen planus (LP) has been documented. In some of the cases with LS first skin lesions resemble those of LP and histology is often not conclusive. An ultrastructural studies provide the evidence of the defects in the basement membrane zone (BMZ) integrity in both disorders. The aim of the study was to compare the alterations of various regions of the BMZ in LS and LP using laser scanning confocal microscopy. Ten cases of LS and five cases of LP were included to this study. Biopsies from patients' skin and a control biopsy from normal human skin were cut into 40 μm thick slides and labeled with antibodies against beta-4 intergin (lamina lucida marker), collagen IV and N-terminal end of collagen VII (lamina densa markers) using routine immunofluorescence. Three-dimensional (3D) reconstruction of various regions of BMZ showed decreased number and size of the dermal papillae in both LS and LP as compared to normal skin. In LS numerous invaginations and holes were present in BMZ at the level of lamina lucida and lamina densa. Computer animation of 3D projection revealed that thickness of lamina densa observed under the light microscopy is an optical artifact dependent on periodical tortion of lamina densa along its axis. Torsions and invaginations of BMZ are equally responsible for phenomenon of artificial reduplication of the lamina densa observed at the ultrastructural level. IF labeling with antibody against N-terminal end of collagen VII – disclosed presence of a large holes in the lamina densa and the presence of granular material in deep dermis suggestive of partial degradation of lamina densa at the level of anchoring fibers. In LP torsions of BMZ along its axis, invaginations and holes were noticed similarly to those observed in LS. In contrast to LS there was absence of the granular material composed of collagen VII in the dermis. 3D reconstruction of BMZ revealed different alterations in LP and LS at the level of collagen VII and its may serve as a valuable investigative tool for differentiation of both entities, especially in the onset of morbid process where is a diagnostic doubt.

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MMP-21, Unlike MMP-26 and MMP-28, is Induced at Early Stages of Melanoma Progression, but Disappears with More Aggressive Phenotype

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The expression pattern of matrix metalloproteinases (MMPs) is frequently altered during malignant transformation. Our objective was to examine the expression of three recently cloned MMPs, MMP-21, MMP-26 and -28, in melanomas *in vivo* and in culture. MMPs-9, -13, and their inhibitor, TIMP-3, were studied as controls based on previous reports on their expression and prognostic significance in melanoma. Immunohistochemical analyses in melanoma specimens from 35 patients were performed. Based on the results of sentinel node biopsies, eighteen samples were from non-metastatic and seventeen from melanomas with nodal micrometastases. MMP-21 was expressed in melanoma cells in 22/35 cases and its expression was stronger in melanoma samples without micrometastases. 7/10 *in situ* melanomas were positive while five nevus samples were negative for MMP-21. MMP-26 and -28 were not generally detected in melanoma or nevus cells. MMP-13 was detected in melanoma cells in 27/35 samples and the number of MMP-13 positive melanoma cells correlated to sentinel node positivity. MMP-9 was expressed by melanoma cells in only 10/35 samples, while positive neutrophils were detected in fourteen and macrophages in nine samples. No correlation of TIMP-3 expression to the status of lymph nodes was found. MMP-21 mRNA was expressed by RT-PCR in Bowes and ML852 melanoma lines while lower levels were apparent also in WM164, WM165, and G361 cells. MMP-26 was detected only at low levels in ML852 and WM165 cells. Our results suggest that expression of MMP-21 may serve as a marker of malignant transformation of melanocytes, but does correlate with prognosis. While the expression of MMPs-7, -9, -26 and -28 does not correlate with the presence of micrometastases, the number of MMP-13 positive cells may correlate to more aggressive disease.

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Antioxidant Enzyme and Methionine Sulfoxide Reductase Expression in Lichen Sclerosus

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Lichen sclerosus (LS) is a chronic inflammatory skin disease of unknown aetiology. The mechanisms which lead to the immunological changes in all levels of the skin and the alterations of the dermal extracellular matrix are poorly understood. We have recently demonstrated an involvement of oxidative stress in the pathogenesis of LS. In this study, we investigated antioxidant defense mechanisms and the expression of the oxidative damage repair enzyme methionine sulfoxide reductase A (MSRA) in LS.

Skin biopsies from 16 patients with untreated, histologically confirmed vulval LS were examined immunohistochemically. Antioxidant enzyme expression of catalase (Cat), copper-zinc superoxide dismutase (CuZnSOD) and manganese SOD (MnSOD) was investigated as well as the distribution of the oxidative damage repair enzyme methionine sulfoxide reductase A (MSRA). Normal vulval tissue from 16 subjects served as control.

In lichen sclerosus tissue the enzymatic antioxidant defense was disturbed as demonstrated by a significantly reduced expression of MnSOD within the stratum corneum and epidermis, and an increased expression of Cat in epidermal and dermal layers. Furthermore, MSRA was shown to be increased in dermal layers of LS lesions suggesting the involvement of a recently described oxidative protein damage repair mechanism.

This is the first study investigating the antioxidant defense and oxidative damage repair capacities in LS. Further studies are required to elucidate the role of the observed modulation in the enzymatic antioxidant system in order to provide new insight into the possibilities of antioxidant therapeutic strategies.

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Withdrawn

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Pathological Changes in Human Skin Caused by Arthropods from Genera *Argas* and *Simulium*

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During the last few years, increased attack frequency of the blood sucking ectoparasites on humans was observed in a vicinity of human habitation in suburban areas of Warsaw in Poland. In this report we present and compare cases of skin changes in humans caused by ectoparasites from genera *Simulium* and *Argas.* 15 persons, 18–60 years old from Warsaw areas with symptoms of arthropod bites, were clinically assessed for pretreatment skin status. All persons examined showed symptoms of dermal alterations of various intensity. However, local skin changes differed depending on kind of causative organism. Black fly bites, usually numerous, resulting in pruritus and surface, small, painful bleeding blisters, caused strong skin reaction, with inflammatory process, damages of the blood capillaries, sometimes with fever and local allergic reaction. In the ultrastructural study, advanced process of rebuilding in the dermal cell region with intensive neoangiogenesis was revealed. The cutaneous changes were healing fully after a symptomatic treatment. *Argas* bites were initially painless, however, they were marked deeper than those of *Simulium* and caused diffuse erythema (oval, round in shape) maintaining during several days. Some sites of the mite bites were susceptible even after many months. As time passed, poor regeneration process appeared; finally, most of the damages scarred. The differences in human reaction after the arthropod bites observed by us are connected, among other, with 1) specific adaptations in mouthparts morphophysiology that enable the arthropods to feed the blood, 2) human immunity status during exposition to the ectoparasite infestation. It should be also taken into consideration, that environmental factors may promote frequent attacks of the hematophagic arthropods on man, and, thus, subsequent serious viral and parasitic disease.

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VEGF and CCL27 Expression in Alopecic Patient Before and After Diphencyprone Treatment: An Immunohistochemical Study

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Alopecia areata is a relatively common inflammatory form of non-scarring hair loss affecting about 2 or 3% of new patients attending dermatology clinics. Vascular endothelial growth factor (VEGF), essential for angiogenesis and vascular permeability, may be responsible for maintaining proper vasculature around hair follicle, moreover a number of studies provide evidence that apoptosis is a central element in the regulation of vascular injury and vascular remodeling. The cutaneous lymphocyte-associated antigen (CLA) and the skin-associated chemokine CCL27 highlight an important role for epithelial cells in controlling homeostatic lymphocyte trafficking.

Topical immunotherapy, using a potent contact allergen such as diphencyprone (DPC), is currently considered the most effective mode of treatment. However, the way in which DPC operates on hair follicles in AA still remains to be elucidated. Therefore the aim of our study was to evaluate the expression pattern of VEGF, Factor VIII, survivin, CD4, CD8, CLA and CCL27 in alopecic skin before and after treatment with DPC.

Our data suggest that topical immunotherapy exert an important role in angiogenesis, upregulating VEGF in human hair follicle keratinocytes and survivin to preserve endothelial cell viability. Moreover it considerably alters the peribulbar CD4/CD8 ratio restoring a condition close to normal scalp skin.