Endothelial cell serine proteases expressed during vascular morphogenesis and angiogenesis

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Summary

Many serine proteases play important regulatory roles in complex biological systems, but only a few have been linked directly with capillary morphogenesis and angiogenesis. Here we provide evidence that serine protease activities, independent of the plasminogen activation cascade, are required for microvascular endothelial cell reorganization and capillary morphogenesis *in vitro*. A homology cloning approach targeting conserved motifs present in all serine proteases, was used to identify candidate serine proteases involved in these processes, and revealed 5 genes (acrosin, testisin, neurosin, PSP and neurotrypsin), none of which had been associated previously with expression in endothelial cells. A subsequent gene-specific RT-PCR screen for 22 serine proteases confirmed expression of these 5 genes and identified 7 additional serine protease genes expressed by human endothelial cells, urokinase-type plasminogen activator, protein C, TMPRSS2, hepsin, matriptase/MT-SP1, dipeptidylpeptidase IV, and seprase. Differences in serine protease gene expression between microvascular and human umbilical vein endothelial cells (HUVECs) were identified and several serine protease genes were found to be regulated by the nature of the substratum, *ie*. artificial basement membrane or fibrillar type I collagen. mRNA transcripts of several serine protease genes were associated with blood vessels *in vivo* by *in situ* hybridization of human tissue specimens. These data suggest a potential role for serine proteases, not previously associated with endothelium, in vascular function and angiogenesis.

Keywords

Serine protease, endothelial cell, tubule morphogenesis, microvascular, angiogenesis

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Introduction

Endothelial cell remodeling and angiogenesis are fundamental to many normal physiological processes (1) and are also key elements in the progression of growth dependent pathologies such as rheumatoid arthritis, psoriasis, arteriosclerosis and cancer (2, 3). Angiogenesis, the process by which new blood vessels are generated from the pre-existing vasculature, occurs in capillary or post-capillary venules associated with the microvascular endothelium. It is a highly regulated and dynamic process requiring extensive changes in endothelial cell function, including changing interactions with the underlying basement membrane, remodeling of and migration through extracellular matrix (ECM), proliferation and differentiation, eventually resulting in the formation of endothelial tubules with patent lumens capable of transporting blood (3). These complex series of events involve a corresponding complex array of newly expressed molecules, amongst which proteolytic enzymes are

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likely to be critical since tissue remodeling events are required for the observed changes in endothelial function. The importance of regulated proteolysis has already been established for cell migration and ECM remodeling (4), activation of growth factors (5) and release of sequestered regulatory molecules (6, 7).

A limited number of proteolytic enzymes have been implicated in angiogenic processes (8). Attention has largely focused on several members of the matrix metalloproteinase (MMP) family (9) and the plasminogen activation cascade, in particular the serine protease, urokinase-type plasminogen activator (uPA) (10, 11). The serine proteases represent a large protease family (12) that is highly conserved within the human genome (13) and amongst species (14). The proteases of this family share a common chemical mechanism of peptide bond hydrolysis, involving a signature catalytic triad of histidine, aspartate and serine amino acids. Members of this family include digestive enzymes (15), blood coagulation and fibrinolytic enzymes (16), glandular kallikreins (17), and granzymes (18). In addition, the newest members of the family are from an emerging group of membrane-associated serine proteases, the type II transmembrane serine proteases (TTSPs) (19) of which enteropeptidase is the archetypal member (20-22). The proteolytic reactions catalyzed by serine proteases often are critical events in a range of physiological and pathological processes that are analogous to many of the functions carried out by endothelial cells, including directed cell migration, limited digestion, protein processing, tissue remodeling, growth control and tubule morphogenesis.

Here we have investigated serine proteases in processes of endothelial cell remodeling and capillary morphogenesis as a first step towards a better understanding of the role of these enzymes in physiological and pathological angiogenesis. Endothelial cells, either cultured *in vitro* on artificial basement membrane (Matrigel) (23) or in three-dimensional gels of fibrillar type I collagen (24), were shown to be dependent on serine protease catalytic activity for normal endothelial cell reorganization and tubule formation. We have identified serine protease genes expressed during these processes and in addition, demonstrate their presence in human tumor tissue vasculature *in vivo*, thus providing a first insight into the potential role of distinct serine proteases in endothelial cell mediated angiogenesis.

Materials and methods

Cell culture

Human neonatal foreskin microvascular endothelial cells and human umbilical vein endothelial cells (HUVECs) were maintained according to the supplier's instructions in EGM-2 MV and EGM-2, respectively (Clonetics, San Diego, CA).

Culture on Matrigel

Endothelial cells were plated at 1.5×10^4 cells/cm^2 (75-80% confluent) in EGM-2 MV (which contains 5% fetal bovine serum) onto precoated Matrigel 24-well plates (BD Biosciences Pharmingen, San Diego, CA). At the indicated times, cells were zinc-formalin fixed, stained with Toluidine Blue (25) and photographed using a digital camera controlled by C-view imaging software (DVC, Austin, TX) or harvested for RNA isolation.

Culture in type I collagen matrix

Rat tail type I collagen was prepared according to Mookhtiar et al. (26). For all collagen matrix experiments, endothelial cell media (EGM-2 MV or EGM-2) was prepared using fetal bovine serum (Clonetics) passed over a gelatin-Sepharose column (Amersham). Collagen was neutralized by combining 40 volumes of type I collagen (3 mg/mL) with 1 volume of 1 N NaOH and 12 volumes of 5× MCDB-131 (Sigma, St. Louis, MO). For two dimensional collagen cultures, 10 cm cell culture plates were coated with a thin layer of neutralized collagen. Endothelial cells were harvested with trypsin-EDTA and plated at 1.5×10^4 cells/cm^2 in media containing 160 nM tetradecanoyl phorbol acetate (PMA), a concentration routinely used with endothelial cells. For three dimensional collagen cultures, one volume of endothelial cells at 5×10^6 cells/mL was added to 9 volumes of neutralized collagen solution, mixed and pipetted into the wells of a 96-well plate at 75 µL/well, allowed to gel for 10 min and fed with EGM-2 MV containing 160 nM PMA. Tubule formation was allowed to progress for 48 h or as otherwise indicated with the daily addition of growth medium. Where indicated 100 µM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) (Sigma), 300 µM 4-(2-aminoethyl)-benzenesulfonamide (AEBS-NH_2) (Aldrich), or 10 µg/mL anti α_2-integrin antibody (Chemicon, Temecula, CA) was added to the media prior to plating the cells. At the conclusion of the assay the cultures were either harvested for RNA extraction, or fixed with zinc-formalin and stained with Toluidine Blue (25) for cytological analysis of tubule formation.

Homology cloning

Total RNA was isolated from cultured endothelial cells using either Trizol Reagent (Gibco-BRL, Grand Island, NY) or RNAeasy kit (Qiagen, Valencia, CA) and reverse transcribed using Superscript II (Gibco-BRL). The resulting cDNA was amplified by polymerase chain reaction (PCR) using two degenerate primers as previously described (27). PCR products ranging from 400 to 550 bp were purified, re-amplified, cloned into pGEM-T Easy (Promega, Madison, WI) and individual clones sequenced.

RT-PCR

Reverse transcription was performed using a SuperScript II kit (Gibco-BRL). PCR was performed using gene-specific primers (Table 1) designed to span at least one intron and nonhomologous sequences amongst serine proteases. Primers also were determined to be sequence specific based on BLAST searches

of the NCBI database. Negative and positive control reactions were performed, respectively, in the absence of template and using 100 pg of plasmid DNA. Cycling conditions were 94 °C for 30 sec followed by 25 to 35 cycles of 94 °C for 30 sec, 55 °C (except for hepsin and Protein C which were at 58 °C) for 30 sec, 72 °C for 45 sec. Reactions were analyzed on 3% agarose/TAE/ethidium bromide gels and representative PCR products from each set of primers were cloned and fully sequenced to verify identity. The PCR cycle numbers and template concentrations were adjusted to reflect subsaturating conditions. Each cDNA template was normalized for actin expression and monitored for the endothelial cell marker CD31 to enable direct comparisons between endothelial cell culture conditions and cell types.

In situ hybridization

Probes used for *in situ* hybridization were cocktails of 2-5 antisense deoxyoligonucleotides or corresponding control sense deoxyoligonucleotides, each 20-35 bases in length (Table 2). Specific sequences were selected utilizing a multiple sequence alignment of 11 human serine protease cDNAs, designed to span intron/exon boundaries, and were verified by exhaustive BLAST-N searches of the entire NCBI database. Each deoxynucleotide was labeled with a 3' digoxigenin (DIG)-dUTP tail using the DIG oligonucleotide Tailing Kit (Roche Molecular Biochemicals, Mannheim, Germany).

Human tissue specimens were collected within 20 minutes of surgical excision and placed in neutral buffered formalin (0.9% NaCl, 4% formaldehyde) for 6-24 h prior to embedding in paraffin. The tissue sections were selected for evidence of increased vascularity by visual inspection following Mayer's hematoxylin and eosin staining and/or staining for the endothelial cell marker CD31. *In situ* hybridization was performed essentially as described (28). Sections were hybridized at 37 °C for 24 h and then washed to a stringency of 0.25X SSC. Signals were detected by tyramide amplification using the TSA-Plus (DNP-AP) Kit (NEN Life Sciences, Boston, MA) after initial incubation for 30 minutes at room temperature with an anti-DIG horseradish peroxidase conjugate. Color development was performed using NBT/BCIP/levamisole. Slides were counterstained with nuclear fast red and eosin to highlight nuclear and cytoplasmic morphologies, respectively. Signal specificity for each antisense probe cocktail was verified by performing comparable hybridization reactions in the presence of the corresponding sense probe cocktail.

Immunohistochemistry

Immunohistochemical detection of endothelial cells on corresponding sections was performed essentially as described (27) using a mouse anti-human antibody against CD31 (DAKO Australia, Sydney). Antibody binding was detected using a horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Pennsylvania, USA) and visualized using 3,3'-diaminobenzidine. Sections were counterstained with Mayer's hematoxylin.

Results

Endothelial cell reorganization on Matrigel is serine protease dependent

Microvascular endothelial cells reorganized into interconnecting cord-like arrays when cultured for 4 h on Matrigel (Fig. 1A). These pronounced cellular organizational changes occurred rapidly and were extensive compared to the same endothelial cells cultured for ten hours in the absence of an added substratum (Fig. 1D). To determine whether serine proteases may be catalytically involved in this process, microvascular endothelial cells were cultured in the presence of the synthetic serine protease inhibitor, AEBSF (4-[2-aminoethyl]-benzenesulfonyl-fluoride). This compound had been used *in vitro* to inhibit completely the known serine proteases, trypsin, plasmin, substilisin, kallikrein, thrombin, tPA, uPA (29, 30) and also in cell culture systems in the 0.5-5.0 mM range to implicate heretofore undefined serine proteases in various biological processing events (31, 32). AEBSF markedly inhibited the Matrigel-induced changes in the endothelial cell cultures, as the cells failed to form interconnecting arrays and more single cells and isolated cords dominated the AEBSF-treated cultures (Fig. 1B). In the presence of an amide derivative of AEBSF, AEBS-NH_2 (4-[2-aminoethyl]-benzenesulfonylamide) which does not function as a serine protease inhibitor, no inhibitory effects on Matrigel-induced microvascular endothelial cell changes were observed (Fig. 1C), indicating that these cell-substratum organizational changes required the activities of serine proteases. The concentration of AEBSF (100 μM) used in these experiments was even lower than used in previous studies (31, 32), and had no effect on adhesion or morphology of the cells cultured in the absence of an added Matrigel substratum (Fig. 1E). As these effects could have been mediated through the PA/plasminogen/plasmin axis which is involved in endothelial cell matrix remodeling, microvascular endothelial cells were treated either with aprotinin, a potent inhibitor of plasmin (33) or the serpin PAI-1, an inhibitor of uPA and tissue-type plasminogen activator (tPA) (34, 35). The Matrigel-induced morphogenic changes were not sensitive to either aprotinin (Fig. 1F), or PAI-1 (data not shown), indicating that microvascular endothelial cells express other serine proteases that regulate microvascular endothelial cell reorganization on Matrigel.

Distinct subsets of serine protease genes expressed by microvascular endothelial cells

To identify serine proteases expressed by microvascular endothelial cells, a reverse transcriptase-polymerase chain reaction (RT-PCR) based homology cloning approach (36) targeting

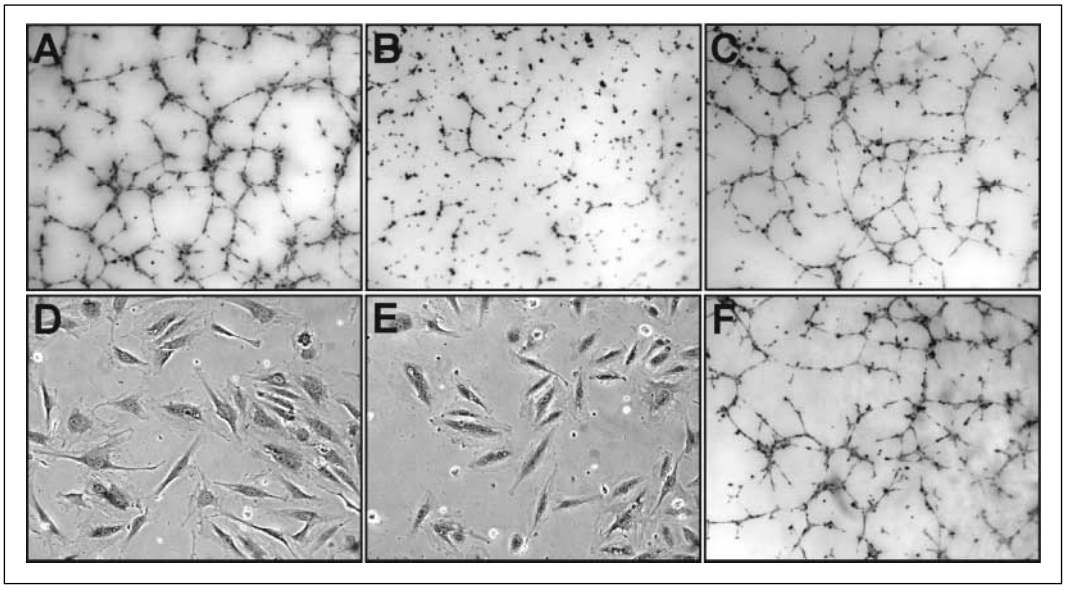

Figure 1: Formation of cord-like structures by human microvascular endothelial cells on Matrigel is serine protease dependent. Microvascular endothelial cells were cultured either on Matrigel coated plastic dishes for 4 h (A-C, F) or in the absence of an added substratum for 10 h (D, E). The cells were either untreated (A, D), or treated with 100 μM AEBSF (B, E), 300 μM AEBS-NH_2 (C) or 300 μM aprotinin (F). Images are of representative fields from each condition. Photomicrographs magnifications are 40X (A-C, F) and 100X (D and E). These data are representative of 3 or more independent experiments.

conserved histidine and serine motifs of the S1 serine protease catalytic triad was employed. cDNAs were synthesized using RNA isolated from microvascular endothelial cells cultured in the presence and absence of Matrigel, and amplified products were cloned and sequenced. Seven different serine protease cDNAs were identified in this screen, including tPA which has been shown previously to be expressed by endothelium (37). Notably, the other 6 serine protease cDNAs identified have not been associated previously with microvascular endothelial cells: neurotrypsin (38), testisin (27), acrosin (39), neurosin (40), CTRL-1 (41), and PSP (putative serine protease; accession number AF193611). While PSP is a member of the serine protease gene family, the deduced protein sequence of PSP lacks structural features required for a catalytically functional serine protease (data not shown).

To verify expression of these mRNAs by microvascular endothelial cells and to screen for additional serine proteases that may not have been targeted by the homology cloning experiments, RT-PCR using gene-specific primers was performed as described in the Materials and Methods with primers listed in Table 1. The expression patterns of a total of 22 serine proteases were investigated in microvascular endothelial cells cultured in the presence and absence of Matrigel. As shown in Figure 2 (left panel), six of the seven serine protease genes identified using the homology cloning approach, were expressed in microvascular endothelial cells cultured on Matrigel or in the absence of an added substratum; expression of only CTRL-1 mRNA could not be verified by gene specific RT-PCR (data not shown). While this RT-PCR technique does not allow absolute quantitation of mRNA levels, three of the serine proteases (acrosin, neurosin and testisin) appeared to be differentially expressed with respect to the presence or absence of the Matrigel substratum, whereas neurotrypsin, tPA and PSP transcript levels were similar under both conditions.

The gene specific RT-PCR screen revealed several additional serine protease genes expressed by microvascular endothelial cells (Fig. 2, right panel). uPA mRNA was expressed by microvascular endothelial cells cultured both in the presence and absence of Matrigel. The mRNA for Protein C, a regulator of hemostasis (42), was detected in microvascular endothelial cells but only following culture on Matrigel. Of the nine type II transmembrane serine proteases (TTSPs) examined, expression of only matriptase (MT-SP1) (36; 43) transcripts was clearly detected, and the signal intensity increased when the endothelial cells were cultured on Matrigel compared with the absence of an added substratum. Two of the TTSPs, TMPRSS2 (44) and hepsin (45), were barely detectable. Other membrane-associated serine protease mRNAs detected included those encoding dipeptidylpeptidase IV (DPP IV) (46), a non-S1 serine protease and Seprase (47), another DPP IV family member (Fig. 2). No products were detected even after extended PCR (35 cycles) using primers specific for CTRL-1, proteinase 3 (48), corin (49), enterokinase (20), HAT (50), MSPL (51), prostasin (52), TMPRSS3 (53), or TMPRSS4 (54) (primers listed in Table 1). These data demonstrate that microvascular endothelial cells can express a limited but substantial number of serine proteases, and that expression of a subset of these genes appear to be regulated by the extracellular substratum.

Table 1: Oligonucleotide primers used for gene specific RT-PCR

Gene[1]	Accession #	5'Primer[2]	3'Primer[2]	Cycles[3]	Product Size[4]
Acrosin	XM_010064	TGTGCATGACTGGAGACTGG	CAGGTGGCCGTGTAGATTCC	35	552
CTRL-1¥	NM_001907	ACTGAGCTTCAGCCAGAGGAT	AGGCTATGACCTGGTTGATCC	35	707
Neurosin	NM_002774	GAATAAGTTGGTGCATGGCG	TCCAGTTCGTGTATCTGCAG	35	653
Neurotrypsin	NM_003619	CTACGCTCCTGAGTAGCTGCTGG	TTGTTGTCATCTCTGCTGAGTGC	30	726
Proteinase 3¥	NM_002777	CAGCACTTCTCGGTGGCTCAG	CAAAGATCCGCCTCGAGGGT	35	546
Protein C	NM_000312	ATGGGAAGATGACCAGGCG	GTCCCATCCATTGCCATGC	35	791
PSP	AF193611	GAACAGTGCTCGGCATGGCA	TGCTCGGCGCGGAACAGT	30	742
Testisin	NM_006799	CTGACTTCCATGCCATCCTT	GCTTCACGACTCCAATCTGAT	35	463
tPA	NM_000931	CCCTGCCTGCTCTGAGGGAAACAG	GAAGAGCCCTCCTTTGATGCGAAA	25	334
uPA	D00244	GTGCATGGTGCATGACTGCGCAGA	AGCGACCCAGGTAGACGATGTAGT	25	290
Corin¥	AF133845	CTTGGAGGTCGGACGAGTCGC	TGGCAAAAGTCTCTGATCATC	35	751
enteropeptidase¥	U09860	TGATATGTGCAGGCTATGAAGAAGG	TTTCCAACAGTGACATACTTTGGG	35	610
HAT¥	AB002134	GTGACTGGATTGCCACGTCTGGT	TAAGACAGGCACACCTGCATACAGAC	35	603
Hepsin	M18930	CCAACAGCGAGGAGAACAGCAAC	GGTCAGAGCTGGGTCACCATGCC	35	511
Matriptase-1/MT-SP1	AF133086	CGTCATCAACCAGACCACCTG	CAGATTTGGAGCCCTGGAGAT	35	410
MSPL¥	AB048796	CCAGCAACCTGCACCAGTTG	GTGTCCTGCTGCAGGGATCTTA	35	546
Prostasin¥	NM_002773	CCTCAGTGAGCCTCCTGACG	CATCCTGGAAGTAGGGCCAGC	35	521
TMPRSS2	U75329	TGCGGGGTCAACTTGAACTC	AAGCTTTGGCACAGCCAGAAC	35	676
TMPRSS3¥	AB038157	CATCCCACTTGGTGGAGAAGAT	CCTCAGGAACTCAGGTGGCTAC	35	555
TMPRSS4¥	AF179224	CAGACAAACTGGGCAGCTTC	AGCTCAGCCTTCCAGACATTG	35	526
DPPIV/CD26	NM_001935	ACCTCAATGGTCCTGGGATCG	CAGGGAATCTATGCAAAGCCTCC	25	703
Seprase/FAP	NM_004460	ACTGGCCCTTGCATCTGGAAC	GATACAGGCTTGCATCTGCATCG	30	417
ß-actin	XM_004814	TGGAGTCCTGTGGCATCCACGAAAC	GCATTTGCGGTGGACGATGGAGGGGCC	23	337
CD31	M28526	CGGAGTGATCATTGCTCTCTTGATC	TCTCGGAACATGGATGTCCTTCCAG	30	439

[1]Genes indicated by ¥ were not detected in any samples examined and are not shown in the figures.
[2]All primers were designed to span an intron to distinguish signals from genomic DNA contaminants.
[3]Number of cycles used in the RT-PCR reactions shown in Figures 2 and 4.
[4]Size in bp of predicted product. All PCR products were the same size as obtained with the EST positive control.

Tubule morphogenesis of endothelial cells in a three dimensional collagen matrix is sensitive to serine protease inhibitors

When microvascular endothelial cells are cultured in a three dimensional type I collagen matrix, they undergo a program of phenotypic changes that leads to multicellular tubule formation within 48 h (Fig. 3A). These vessel-like multicellular structures are true endothelial cell tubules with a matrix-free lumen (inset Fig. 3A). When collagen-embedded microvascular endothelial cells were cultured in the presence of 100 µM AEBSF, multicellular tubule formation was inhibited (Fig. 3B). The control compound AEBS-NH_2 did not inhibit tubule formation (Fig. 3D), indicating again that serine protease activities are

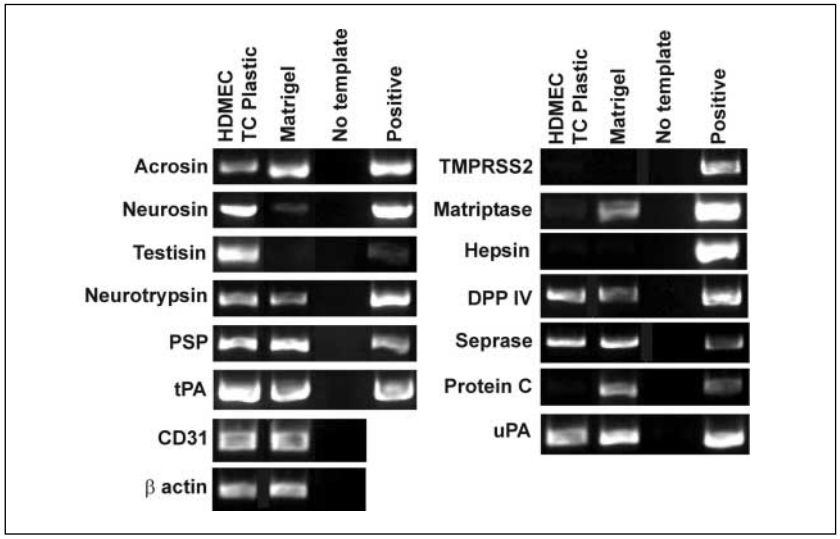

Figure 2: Gene specific RT-PCR analysis of serine protease mRNA expression by microvascular endothelial cells (HDMEC) cultured on Matrigel. Total RNA was isolated from human microvascular endothelial cells cultured either in the absence of a substratum (TC Plastic) or on Matrigel coated plastic dishes for 10 h. Plasmids containing ESTs encoding each serine protease were used as templates (100 pg cDNA) in positive control reactions. Negative control reactions were performed in the absence of template.

Table 2: Oligonucleotide probe cocktails used for *in situ* hybridization[1]

Neurotrypsin	
AS3:	5'AGTCCACCTTGCCACGGGCGTCTCCGTAGAAACAC
AS7:	5'CTTCATCTCCTCGGCAACGGACATT
AS13:	5'TTAACCACTGATTTCCTCACTTGACAAAGGCTACA
AS18:	5'ATGTGGGTATTTAATATGATTTCAGACACCACTGA
Acrosin	
AS1:	5'TTTAGCAACCACGGACACTGCCAAGACCAGCAGAA
AS2:	5'GGGCTCACTGGGTCTGTTGAGAACCAGGTCAGATC
AS4:	5'CTCTGTGGTCTCCATGTCATAATGGTTCTTTCCGT
AS5:	5'TACCAAGGAAGGTGAGCAGAGATAGGGTGGGAGAA
Hepsin	
AS1	5'CCTGAGGAGAACAGCCACAA
AS2	5'TCACACACGGAGATACCTCC
AS3	5'CAGGCTGGATGTATTCTGTG
AS4	5'TCGGAGTGAGTCTTTATGGC
Matriptase-1	
AS2:	5'ATGACGCTGCCCTCGCTGA
AS3:	5'GTTGTTGGGCACCTCAATGT
AS5:	5'AGTGCCTCCATACTGGGTGT
AS6:	5'GAATCACCCTGGCAGGAGTC
TMPRSS2	
AS1:	TGGTGACCCTGAGTTCAAAG
AS2:	AGAGGCGAACACACCGATTC
AS3:	TCACTAGGTCGTTGAAAGTC
AS4:	TCTGAGGTCTTCCCTTTCTC
AS5:	CACTGTCACCCTGGCAAGAA
Neurosin	
AS8:	GGAGGCAAGGTCTAGGTGAGAGACGTTCTGGAACC
AS9:	GTGAGATGTCTTGTCGCAGGGTCCGCCATGCACC

[1]Sequences of antisense primers are listed. Sense primers consisted of the reverse complement of each of these sequences.

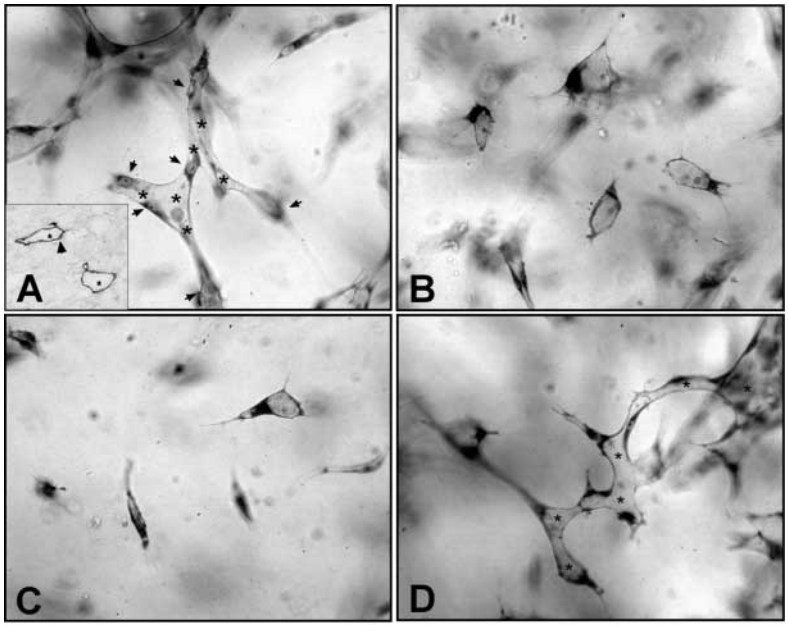

Figure 3: *In vitro* tubular morphogenesis of microvascular endothelial cells is serine protease dependent. Human microvascular endothelial cells were allowed to undergo tubule morphogenesis in a 3-D type I collagen matrix (A). Tubule formation was blocked in the presence of 100 μM AEBSF (B), 10 μg/mL anti-α_2 integrin antibody (C), but not in the presence of 300 μM AEBS-NH_2 (D). Inset in panel A shows cross-sections of tubules. The * mark lumens and the arrowheads indicate endothelial cells. Cells were cultured for 96 h and then visualized by phase-contrast microscopy and photographed at 100X magnification. These data are representative of 3 or more independent experiments.

involved in these endothelial differentiation processes. A similar inhibition of multicellular tubule formation was observed using a specific function-blocking anti-α_2-integrin antibody (24) as shown in Figure 3c; tubule formation also was insensitive to the plasmin inhibitor aprotinin (data not shown) which is consistent with the findings of Davis et al. who showed that plasminogen (plasmin) is not involved in tube formation but contributes to vascular regression (55). These data, showing blockage of tubule formation by AEBSF and not by AEBS-NH_2, demonstrate a functional role for serine proteases in endothelial cell tubule morphogenesis. This serine protease dependence is apparently not unique to the microvasculature as human umbilical vein endothelial cells (HUVECs), a type of specialized 'macrovascular' endothelial cell, showed a similar morphological phenotype and displayed the same sensitivity to AEBSF as microvascular endothelial cells (data not shown).

Serine proteases are differentially expressed by endothelial cells during tubule morphogenesis

To identify serine proteases that may be important for vascular tubule formation, microvascular endothelial cells and HUVECs were allowed to undergo tubule morphogenesis within a three dimensional type I collagen matrix and were compared with endothelial cells cultured on the surface of a thin layer of type I collagen (two dimensional collagen). RNA was isolated and RT-PCR performed as above using gene specific primers (Table 1) to investigate the expression patterns of the 22 serine proteases. Three independent experiments gave similar results and are represented in Figure 4. Most of the serine protease transcripts detected in microvascular endothelial cells cultured on Matrigel (Fig. 2) were also expressed by endothelial cells cultured within and on type I collagen, with the exception of protein C (Fig. 4). Neurosin and acrosin mRNAs were detected in both microvascular endothelial cells and HUVECs, and their expression appeared to be up-regulated in the microvascular endothelial cells cultured in three dimensional collagen. tPA, uPA and PSP transcripts were strongly expressed under all conditions. Interestingly, neurotrypsin transcripts were detected in microvascular endothelial cell cultures, but were not present in HUVECs indicating a cell-type specificity for this serine protease.

Of the membrane-associated serine proteases, TMPRSS2 mRNA showed a strong signal in microvascular endothelial cells cultured only in three dimensional collagen, and thus appears to be specifically induced as a response to collagen-induced tubule morphogenesis. Testisin transcripts were more strongly detected in microvascular endothelial cells than in HUVECs, and the levels did not change significantly during tubule morphogenesis in three dimenstional collagen. Signals for hepsin were weak overall, but were more associated with HUVECs. DPP IV mRNA expression was detected more strongly in microvascular endothelial cells, while seprase was expressed in both microvascular endothelial cells and HUVECs. Matriptase mRNA was detectable only at very low levels in both microvascular endothelial cells and HUVECs cultured in the presence of collagen (Fig. 4), in contrast to its relatively strong expression by microvascular endothelial cells cultured on Matrigel (Fig. 2). These data demonstrate a number of serine proteases expressed by microvascular endothelial cells, several of which appear to be regulated not only by the surrounding cell substratum but also in a cell-type specific manner.

Serine protease mRNAs are expressed by human endothelium *in vivo*

To determine whether some of the serine protease genes detected in the *in vitro* endothelial cell models were expressed by human endothelium *in vivo*, a selection of human tissue speci-

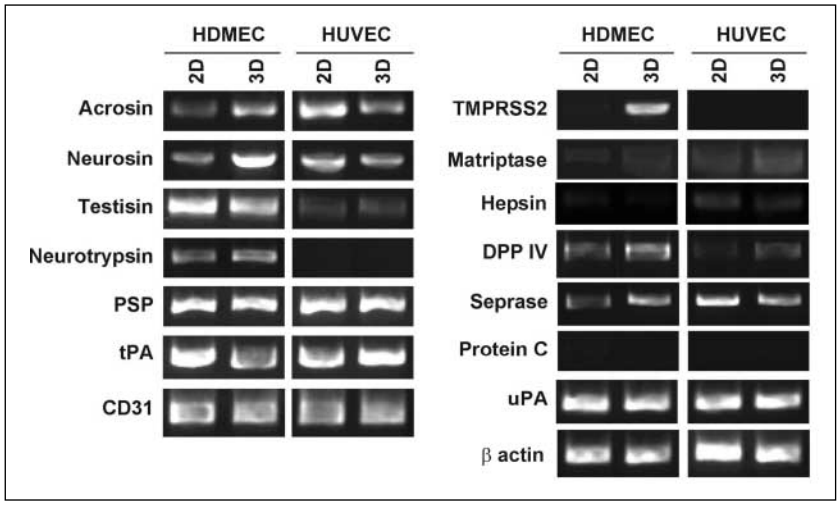

Figure 4: Gene specific RT-PCR reveals differential mRNA expression of serine proteases in endothelial cells undergoing tubule morphogenesis in a 3-D type I collagen matrix. Human dermal microvascular endothelial cells (HDMEC) and HUVECs were allowed to undergo tubule morphogenesis in a 3-D type I collagen matrix (3D) and compared with endothelial cells cultured on the surface of a thin layer of type I collagen (2D). After 48 h the cells were harvested, total RNA isolated, and analyzed by gene specific RT-PCR. Positive and negative control reactions were performed as described in Fig. 2 and are not shown.

mens was examined for evidence of serine protease mRNA expression by *in situ* hybridization (Fig. 5). Endothelial cells present in the tissue vasculature were confirmed by immunohistochemical staining for the endothelial cell marker, CD31. Intense positive staining for neurotrypsin mRNA was detected throughout the vasculature of a section of adenocarcinomatous metastasis in brain (Fig. 5a). No staining was evident when the sense probe was employed (Fig. 5b). The antisense staining pattern correlated with anti-CD31 staining (Fig. 5c), showing that neurotrypsin expression was endothelial cell associated. Similarly, strong specific staining for acrosin mRNA was associated with endothelial cells present in the vasculature of a benign acoustic neuroma (Fig. 5d), compared with the sense control (Fig. 5e). The staining appeared to be associated with smooth muscle cells as well as with endothelial cells, showing a pattern that correlated well with CD31 staining (Fig. 5f). *In situ* hybridization signals for other serine protease mRNAs were variable amongst tissue specimens. Specific staining for hepsin mRNA was detected in some endothelial cells associated with a group of vessels present in a gastro-esophageal junctional adenocarcinoma (Fig. 5g vs 5h). The corresponding pattern of anti-CD31 staining indicated that hepsin expression was associated with the endothelial cells present in the vasculature (Fig. 5i). *In situ* hybridization using antisense probes for TMPRSS2, matriptase and neurosin yielded diffuse perivascular staining (data not shown) but were not resolved sufficiently to indicate definitive endothelial cell staining. These *in situ* hybridization data confirm expression of several serine protease genes, not previously associated with endothelial cells, within the vasculature of human tissues *in vivo*, and suggest expression may be associated only with specific vascular endothelium.

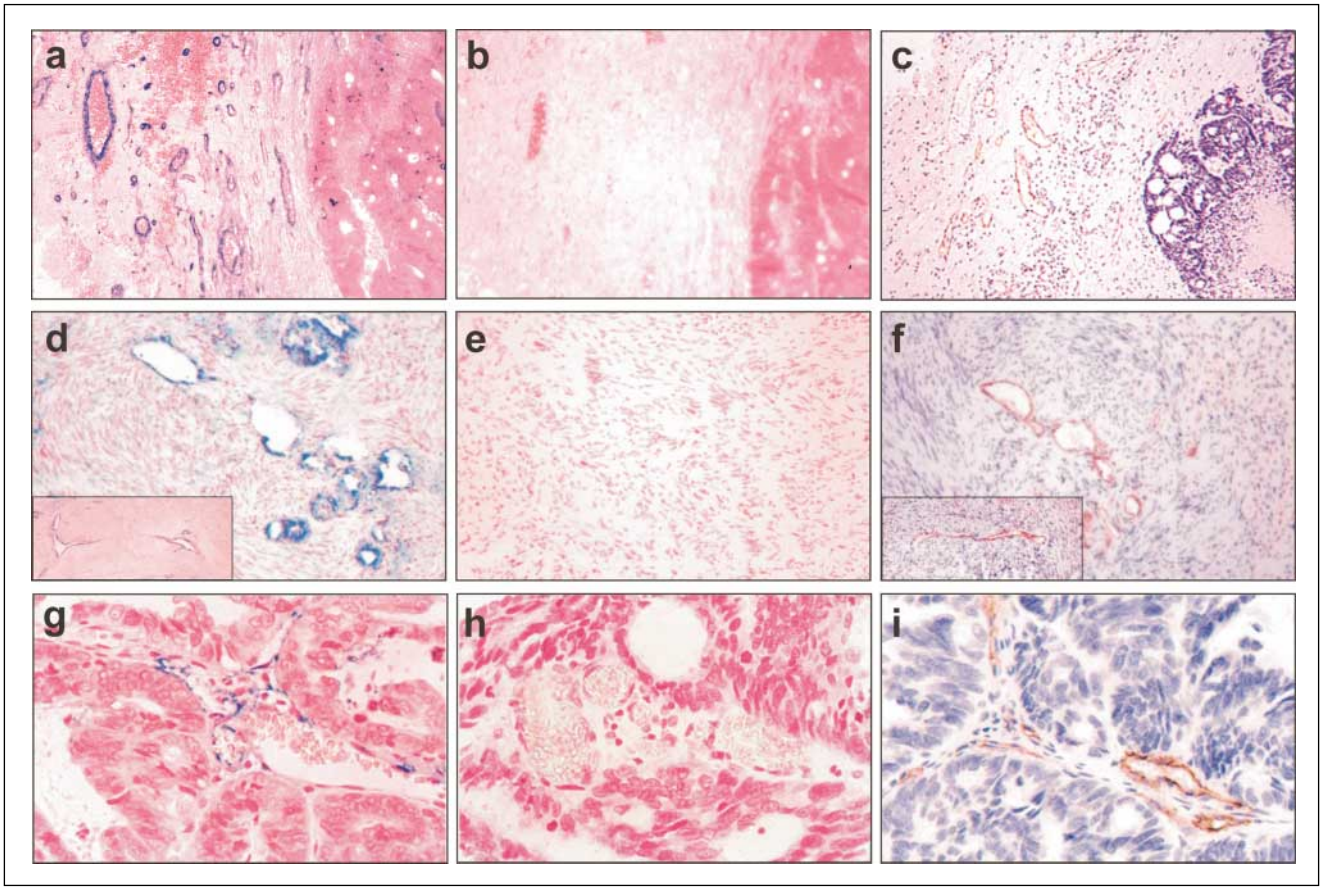

Figure 5: Photomicrographs of mRNA expression of several serine proteases in human vasculature by *in situ* hybridization. Expression of neurotrypsin mRNA in endothelial cells associated with the vasculature of an adenocarcinomatous metastases in brain at 100x original magnification (a) antisense probe (b) sense control (c) anti-CD31 staining. Acrosin mRNA expression in the vasculature of a benign acoustic neuroma at 200x original magnification, (d) antisense probe (e) sense control probe (f) anti-CD31 staining. Insets in (d) and (f) show serial sections at 40x original magnification further illustrating the corresponding pattern of acrosin antisense and CD31 staining. Hepsin mRNA expression in a section of gastro-esophageal junction adenocarcinoma at 400x original magnification, (g) antisense hepsin probe (h) sense control probe (i) anti-CD31 localization of vessel endothelium. Antisense probes show blue tyramide amplification of positive signals. Nuclei are stained red and orange indicates cytoplasmic stain that highlights the erythrocytes present in some vessels. Immunostaining with anti-CD31 is brown with contrasting blue staining of nuclei.

Discussion

We have demonstrated that *in vitro* endothelial cell reorganization and tubule formation is serine protease dependent and have and identified 13 known members in the serine protease family that are expressed during these processes, 6 of which appear to be differentially expressed depending on cell type or cell substratum. Furthermore, we have provided evidence for expression of a number of serine protease genes in human vasculature *in vivo*, underlining the potential of serine proteases to mediate endothelial cell function crucial for angiogenesis and angiostasis.

While others have reported that inhibitors which prevent direct interaction of endothelial cells with key matrix components (23, 24, 56-58), or prevent proper processing of the extracellular matrix (11; 59) clearly disrupt the formation of vessel-like structures *in vitro*, this is the first time that AEBSF-sensitive serine proteases, independent of the plasminogen activation system, have been implicated in endothelial cell morphogenesis and neovascularization. As angiogenesis is a dynamic process, endothelial cells may require differential proteolytic capabilities at different stages during vascular tubule morphogenesis. Expression of the 13 serine protease genes varied depending on whether or not the endothelial cells were of microvascular origin (HDMECs vs HUVECs). Furthermore the type of extracellular matrix used to facilitate tubule morphogenesis had a significant impact on serine protease gene expression, indicating that the full endothelial cell serine protease repertoire may be modified in direct response to the microenvironment. Indeed, *in situ* hybridization experiments frequently showed variable staining of tissue vasculature, further suggesting that the local tissue microenvironment can influence serine protease expression. It might now be predicted that serine protease expression during development of new vessels is regulated spatially and temporally, analogous to the control of other endothelial regulatory genes (e.g., vWF, endoglin, and VEGF-R2) (60-64).

The *in vitro* and *in vivo* expression data presented here highlight several serine protease genes that have been reported previously to have very restricted tissue expression; for example, acrosin to the sperm acrosome (65), testisin to eosinophils (66) and premeiotic male germ cells (27), hepsin to liver and kidney (67) and neurotrypsin to brain neurons (68). mRNA transcripts for neurosin, known as Zyme, Protease M, and human kallikrein-6 (69), also were detected in the endothelial cells. It may be of note that this serine protease has been associated with ovarian and breast cancer and may serve as a serum marker for diagnosing ovarian carcinomas (70). None of these serine proteases had been linked previously to vascular tissue nor reported to be expressed by endothelial cells. The finding that these generally tissue-specific serine proteases are expressed by endothelial cells was unexpected. Unfortunately, little is known about the natural substrates and activators for these enzymes in the various tissues. Hence how these serine proteases contribute to endothelial cell reorganization will be difficult to assess without highly specific reagents such as unique inhibitors, substrates and activators.

Several coagulation and fibrinolysis associated genes were identified (uPA, tPA, Protein C, hepsin and matriptase), suggesting a more complex role of the fibrinolytic cascade during capillary morphogenesis. The up-regulation of Protein C and matriptase mRNAs in microvascular endothelial cells cultured on Matrigel may suggest a functional role for these proteases in endothelial cell migration and organization on basement membranes. Activated protein C functions as an anticoagulant on the surface of endothelial cells (71) while hepsin can activate factor VII, which potentially could lead to thrombin formation (72). uPA and tPA, besides their role in fibrinolysis, also have established roles in ECM remodeling through the generation of plasmin, which can function as a potent remodeler of the ECM and activator of a number of MMP zymogens (73). Matriptase has been reported to activate protease activated receptor-2 (PAR2), hepatocyte growth factor (HGF) as well as pro-uPA (74, 75).

Genes encoding 5 integral membrane serine proteases were expressed by cultured endothelial cells: matriptase, hepsin, and TMPRSS2, seprase and DPP IV. The location of seprase at the invasive front of cells and its ability to degrade denatured collagen (76) may facilitate endothelial cell migration. DPP IV is a cell surface receptor for plasminogen-2 and collagen and may serve as both an adhesive molecule and to focus proteolytic activity to the endothelial cell membrane (77). Particularly intriguing was the expression profile of TMPRSS2, which was up-regulated only in microvascular endothelial cells induced to undergo tubule formation in three dimensional collagen. It is interesting to speculate that TMPRSS2 function may be restricted only to endothelial cells actively undergoing an angiogenic or tubulogenic response. In addition to their serine protease activities, integral membrane serine proteases also have the potential to serve as endothelial cell receptors and/or adhesion molecules via their catalytic as well as their extracellular protein binding domains (19).

While extensive literature exists on the roles of uPA, tPA and the plasminogen/plasmin system in endothelial cell behavior (10), the functions of most of the other serine proteases identified *in vivo* are poorly understood. There are many vascular processes that may well depend on serine proteases activities. In addition to ECM remodeling and proteolytic activation/inactivation of growth factors and cytokines, release of bioavailable molecules and exposure of neo-epitopes after proteolytic attack could all constitute unique signals to trigger stages in angiogenesis and vascular tubule formation. Several of these serine proteases, and in particular the TTSPs, are multidomain proteins with potential for serving as transducers of

intracellular and extracellular signals (19). Proteolytic release of some of these domains from the cell surface, as has been demonstrated for TMPRSS2 (78), may also provide additional signals to the vasculature. It is not possible at present to determine clearly how many of the 13 expressed serine protease genes are translated, activated and catalytically involved in endothelial function. Antibodies that specifically identify the cellular translated enzymes are either not available or lack in selectivity for the individual serine proteases. We attempted to procure specific antibodies to all of the serine proteases whose transcripts were identified. Although most of them were not commercially available, many of the antibodies acquired from various laboratories yielded multiple cross-reactive bands in Western blots of endothelial cell lysates (data not shown), and therefore could not be used as definitive immunological probes for the presence of the translated serine proteases. The few of the serine proteases that were already known to be expressed by endothelial cells (e.g. uPA, tPA) were immunologically detectable (data not shown). Selective substrates and/or inhibitors that could distinguish activities of the multiple transcript-detected serine proteases also are not available and thus elucidation of the catalytic role for these endothelial cell serine proteases is not possible at the present time. It will be important in future to generate antibodies and inhibitors specific for these serine proteases to investigate their various biological activities during endothelial cell morphogenesis. For the present, this study represents the first report to implicate directly serine protease activity in endothelial morphogenesis using a more general serine protease inhibitor and to identify a subset of viable candidate enzymes that had never been linked to vascular function previously. Clearly these proteases represent an interesting class of genes that may provide new and exciting targets for therapeutic intervention in neovasculargenesis and vascular remodeling.

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References

1. Risau W. Mechanisms of angiogenesis. Nature 1997; 386 (6626): 671-4.
2. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1995; 1 (1): 7-31.
3. Carmeliet P, Collen D. Transgenic mouse models in angiogenesis and cardiovascular disease. J Pathol 2000; 190 (3): 387-405.
4. Mignatti P, Rifkin DB. Biology and biochemistry of proteinases in tumor invasion. Physiol Rev 1993; 73: 161-95.
5. Rifkin DB, Mazzieri R, Munger JS, Noguera I, Sung J. Proteolytic control of growth factor availability. APMIS 1999; 107 (1): 80-5.
6. Plouet J, Moro F, Bertagnolli S, Coldeboeuf N, Mazarguil H, Clamens S et al. Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form by urokinase is required for its mitogenic effect. J Biol Chem 1997; 272 (20): 13390-6.
7. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2000; 2 (10): 737-44.
8. Pepper MS. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler Thromb Vasc Biol 2001; 21 (7): 1104-17.
9. Werb Z, Vu TH, Rinkenberger JL, Coussens LM. Matrix-degrading proteases and angiogenesis during development and tumor formation. APMIS 1999; 107 (1): 11-8.
10. Pepper MS, Montesano R, Mandriota SJ, Orci L, Vassalli J-D. Angiogenesis: a paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. Enzyme Protein 1996; 49 (1-3): 138-62.
11. van Hinsbergh VW, Koolwijk P, Hanemaaijer R. Role of fibrin and plasminogen activators in repair-associated angiogenesis: in vitro studies with human endothelial cells. EXS 1997; 79: 391-411.
12. Rawlings ND, Barrett AJ. Families of serine peptidases. Methods Enzymol 1994; 244: 19-61.
13. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J et al. Initial sequencing and analysis of the human genome. Nature 2001; 409 (6822): 860-921.
14. Krem MM, Rose T, Di Cera E. Sequence determinants of function and evolution in serine proteases. Trends Cardiovasc Med 2000; 10 (4): 171-6.
15. Neurath H. Proteolytic enzymes, past and present. Fed Proc 1985; 44 (14): 2907-13.
16. Degen JL, Drew AF, Palumbo JS, Kombrinck KW, Bezerra JA, Danton MJ et al. Genetic manipulation of fibrinogen and fibrinolysis in mice. Ann N Y Acad Sci 2001; 936: 276-90.
17. Clements J, Hooper J, Dong Y, Harvey T. The expanded human kallikrein (KLK) gene family: genomic organisation, tissue-specific expression and potential functions. Biol Chem 2001; 382 (1): 5-14.
18. Kam CM, Hudig D, Powers JC. Granzymes (lymphocyte serine proteases): characterization with natural and synthetic substrates and inhibitors. Biochim Biophys Acta 2000; 1477 (1-2): 307-23.
19. Hooper JD, Clements JA, Quigley JP, Antalis TM. Type II transmembrane serine proteases. Insights into an emerging class of cell surface proteolytic enzymes. J Biol Chem 2001; 276 (2): 857-60.
20. Kitamoto Y, Veile RA, Donis-Keller H, Sadler JE. cDNA sequence and chromosomal localization of human enterokinase, the proteolytic activator of trypsinogen. Biochemistry 1995; 34 (14): 4562-8.
21. Mann NS, Mann SK. Enterokinase. Proc Soc Exp Biol Med 1994; 206 (2): 114-8.
22. Kitamoto Y, Yuan X, Wu Q, McCourt DW, Sadler JE. Enterokinase, the initiator of intestinal digestion, is a mosaic protease composed of a distinctive assortment of domains. Proc Natl Acad Sci USA 1994; 91 (16): 7588-92.
23. Kubota Y, Kleinman HK, Martin GR, Lawley TJ. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol 1988; 107 (4): 1589-98.
24. Davis GE, Camarillo CW. An alpha 2 beta 1 integrin-dependent pinocytic mechanism in-

volving intracellular vacuole formation and coalescence regulates capillary lumen and tube formation in three-dimensional collagen matrix. Exp Cell Res 1996; 224 (1): 39-51.
25. Yang S, Graham J, Kahn JW, Schwartz EA, Gerritsen ME. Functional roles for PECAM-1 (CD31) and VE-cadherin (CD144) in tube assembly and lumen formation in three-dimensional collagen gels. Am J Pathol 1999; 155 (3): 887-95.
26. Mookhtiar KA, Mallya SK, Van Wart HE. Properties of radiolabeled type I, II, and III collagens related to their use as substrates in collagenase assays. Anal Biochem 1986; 158 (2): 322-33.
27. Hooper JD, Nicol DL, Dickinson JL, Eyre HJ, Scarman AL, Normyle JF et al. Testisin, a new human serine proteinase expressed by premeiotic testicular germ cells and lost in testicular germ cell tumors. Cancer Res 1999; 59 (13): 3199-205.
28. Tata AM. An in situ hybridization protocol to detect rare mRNA expressed in neural tissue using biotin-labelled oligonucleotide probes. Brain Res Brain Res Protoc 2001; 6 (3): 178-84.
29. Mintz GR. An irreversible serine protease inhibitor. Biopharmacology 1993; 6: 34-38.
30. Chu TM, Kawinski E. Plasmin, substilisin-like endoproteases, tissue plasminogen activator, and urokinase plasminogen activator are involved in activation of latent TGF-beta 1 in human seminal plasma. Biochem Biophys Res Commun 1998; 253 (1): 128-34.
31. Bestilny LJ, Riabowol KT. A role for serine proteases in mediating phorbol ester-induced differentiation of HL-60 cells. Exp Cell Res 2000; 256 (1): 264-71.
32. Citron M, Diehl TS, Capell A, Haass C, Teplow DB, Selkoe DJ. Inhibition of amyloid beta-protein production in neural cells by the serine protease inhibitor AEBSF. Neuron 1996; 17 (1): 171-9.
33. Fussi F. Aprotinin: mechanism of action and therapeutical uses. Boll Chim Farm 1980; 119 (11): 631-46.
34. Andreasen PA, Nielsen LS, Kristensen P, Grondahl-Hansen J, Skriver L, Dano K. Plasminogen activator inhibitor from human fibrosarcoma cells binds urokinase-type plasminogen activator, but not its proenzyme. J Biol Chem 1986; 261 (17): 7644-51.
35. Loskutoff DJ, Edgington TS. An inhibitor of plasminogen activator in rabbit endothelial cells. J Biol Chem 1981; 256 (9): 4142-5.
36. Takeuchi T, Shuman MA, Craik CS. Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. Proc Natl Acad Sci USA 1999; 96 (20): 11054-61.
37. Bykowska K, Levin EG, Rijken DC, Loskutoff DJ, Collen D. Characterization of a plasminogen activator secreted by cultured bovine aortic endothelial cells. Biochim Biophys Acta 1982; 703 (1): 113-5.
38. Proba K, Gschwend TP, Sonderegger P. Cloning and sequencing of the cDNA encoding human neurotrypsin. Biochim Biophys Acta 1998; 1396 (2): 143-7.
39. Baba T, Watanabe K, Kashiwabara S, Arai Y. Primary structure of human proacrosin deduced from its cDNA sequence. FEBS Lett 1989; 244 (2): 296-300.
40. Yamashiro K, Tsuruoka N, Kodama S, Tsujimoto M, Yamamura Y, Tanaka T et al. Molecular cloning of a novel trypsin-like serine protease (neurosin) preferentially expressed in brain. Biochim Biophys Acta 1997; 1350 (1): 11-4.
41. Reseland JE, Larsen F, Solheim J, Eriksen JA, Hanssen LE, Prydz H. A novel human chymotrypsin-like digestive enzyme. J Biol Chem 1997; 272 (12): 8099-104.
42. Foster D, Davie EW. Characterization of a cDNA coding for human protein C. Proc Natl Acad Sci USA 1984; 81 (15): 4766-70.
43. Lin CY, Anders J, Johnson M, Dickson RB. Purification and characterization of a complex containing matriptase and a Kunitz-type serine protease inhibitor from human milk. J Biol Chem 1999; 274 (26): 18237-42.
44. Paoloni-Giacobino A, Chen H, Peitsch MC, Rossier C, Antonarakis SE. Cloning of the TMPRSS2 gene, which encodes a novel serine protease with transmembrane, LDLRA, and SRCR domains and maps to 21q22.3. Genomics 1997; 44 (3): 309-20.
45. Leytus SP, Loeb KR, Hagen FS, Kurachi K, Davie EW. A novel trypsin-like serine protease (hepsin) with a putative transmembrane domain expressed by human liver and hepatoma cells. Biochemistry 1988; 27 (3): 1067-74.
46. Darmoul D, Lacasa M, Baricault L, Marguet D, Sapin C, Trotot P et al. Dipeptidyl peptidase IV (CD 26) gene expression in enterocyte-like colon cancer cell lines HT-29 and Caco-2. Cloning of the complete human coding sequence and changes of dipeptidyl peptidase IV mRNA levels during cell differentiation. J Biol Chem 1992; 267 (7): 4824-33.
47. Goldstein LA, Ghersi G, Pineiro-Sanchez ML, Salamone M, Yeh Y, Flessate D et al. Molecular cloning of seprase: a serine integral membrane protease from human melanoma. Biochim Biophys Acta 1997; 1361 (1): 11-9.
48. Campanelli D, Melchior M, Fu Y, Nakata M, Shuman H, Nathan C et al. Cloning of cDNA for proteinase 3: a serine protease, antibiotic, and autoantigen from human neutrophils. J Exp Med 1990; 172 (6): 1709-15.
49. Yan W, Sheng N, Seto M, Morser J, Wu Q. Corin, a mosaic transmembrane serine protease encoded by a novel cDNA from human heart. J Biol Chem 1999; 274 (21): 14926-35.
50. Yamaoka K, Masuda K, Ogawa H, Takagi K, Umemoto N, Yasuoka S. Cloning and characterization of the cDNA for human airway trypsin-like protease. J Biol Chem 1998; 273 (19): 11895-901.
51. Kim DR, Sharmin S, Inoue M, Kido H. Cloning and expression of novel mosaic serine proteases with and without a transmembrane domain from human lung. Biochim Biophys Acta 2001; 1518 (1-2): 204-9.
52. Yu JX, Chao L, Chao J. Molecular cloning, tissue-specific expression, and cellular localization of human prostasin mRNA. J Biol Chem 1995; 270 (22): 13483-9.
53. Scott HS, Kudoh J, Wattenhofer M, Shibuya K, Berry A, Chrast R et al. Insertion of beta-satellite repeats identifies a transmembrane protease causing both congenital and childhood onset autosomal recessive deafness. Nat Genet 2001; 27 (1): 59-63.
54. Wallrapp C, Hahnel S, Muller-Pillasch F, Burghardt B, Iwamura T, Ruthenburger M et al. A novel transmembrane serine protease (TMPRSS3) overexpressed in pancreatic cancer. Cancer Res 2000; 60 (10): 2602-6.
55. Davis GE, Pintar Allen KA, Salazar R, Maxwell SA. Matrix metalloproteinase-1 and -9 activation by plasmin regulates a novel endothelial cell-mediated mechanism of collagen gel contraction and capillary tube regression in three-dimensional collagen matrices. J Cell Sci 2001; 114 (Pt 5): 917-30.
56. Bahou WF, Potter CL, Mirza H. The VLA-2 (alpha 2 beta 1) I domain functions as a ligand-specific recognition sequence for endothelial cell attachment and spreading: molecular and functional characterization. Blood 1994; 84 (11): 3734-41.
57. Senger DR, Claffey KP, Benes JE, Perruzzi CA, Sergiou AP, Detmar M. Angiogenesis promoted by vascular endothelial growth factor: regulation through alpha1beta1 and alpha2beta1 integrins. Proc Natl Acad Sci USA 1997; 94 (25): 13612-7.
58. Grant DS, Tashiro K, Segui-Real B, Yamada Y, Martin GR, Kleinman HK. Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. Cell 1989; 58 (5): 933-43.
59. Seandel M, Noack-Kunnmann K, Zhu D, Aimes RT, Quigley JP. Growth factor-induced angiogenesis in vivo requires specific cleavage of fibrillar type I collagen. Blood 2001; 97 (8): 2323-32.
60. Bell SE, Mavila A, Salazar R, Bayless KJ, Kanagala S, Maxwell SA et al. Differential gene expression during capillary morphogenesis in 3D collagen matrices: regulated expression of genes involved in basement membrane matrix assembly, cell cycle progression, cellular differentiation and G-protein signaling. J Cell Sci 2001; 114 (Pt 15): 2755-73.
61. Kahn J, Mehraban F, Ingle G, Xin X, Bryant JE, Vehar G et al. Gene expression profiling in an in vitro model of angiogenesis. Am J Pathol 2000; 156 (6): 1887-900.
62. Zanetta L, Marcus SG, Vasile J, Dobryansky M, Cohen H, Eng K et al. Expression of Von Willebrand factor, an endothelial cell marker, is up-regulated by angiogenesis factors: a potential method for objective assessment of tumor angiogenesis. Int J Cancer 2000; 85 (2): 281-8.

63. Fonsatti E, Del Vecchio L, Altomonte M, Sigalotti L, Nicotra MR, Coral S et al. Endoglin: An accessory component of the TGF-beta-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. J Cell Physiol 2001; 188 (1): 1-7.
64. Ortega N, Hutchings H, Plouet J. Signal relays in the VEGF system. Front Biosci 1999; 4: D141-D152.
65. Kashiwabara S, Baba T, Takada M, Watanabe K, Yano Y, Arai Y. Primary structure of mouse proacrosin deduced from the cDNA sequence and its gene expression during spermatogenesis. J Biochem (Tokyo) 1990; 108 (5): 785-91.
66. Inoue M, Kanbe N, Kurosawa M, Kido H. Cloning and tissue distribution of a novel serine protease esp-1 from human eosinophils. Biochem Biophys Res Commun 1998; 252 (2): 307-12.
67. Tsuji A, Torres-Rosado A, Arai T, Le Beau MM, Lemons RS, Chou SH et al. Hepsin, a cell membrane-associated protease. Characterization, tissue distribution, and gene localization. J Biol Chem 1991; 266 (25): 16948-53.
68. Gschwend TP, Krueger SR, Kozlov SV, Wolfer DP, Sonderegger P. Neurotrypsin, a novel multidomain serine protease expressed in the nervous system. Mol Cell Neuorosci 1997; 9 (3): 207-19.
69. Yousef GM, Luo LY, Scherer SW, Sotiropoulou G, Diamandis EP. Molecular characterization of zyme/protease M/neurosin (PRSS9), a hormonally regulated kallikrein-like serine protease. Genomics 1999; 62 (2): 251-9.
70. Diamandis EP, Yousef GM, Soosaipillai AR, Bunting P. Human kallikrein 6 (zyme/protease M/neurosin): a new serum biomarker of ovarian carcinoma. Clin Biochem 2000; 33 (7): 579-83.
71. Esmon CT. The anticoagulant and anti-inflammatory roles of the protein C anticoagulant pathway. J Autoimmun 2000; 15 (2): 113-6.
72. Kazama Y, Hamamoto T, Foster DC, Kisiel W. Hepsin, a putative membrane-associated serine protease, activates human factor VII and initiates a pathway of blood coagulation on the cell surface leading to thrombin formation. J Biol Chem 1995; 270 (1): 66-72.
73. Ramos-DeSimone N, Hahn-Dantona E, Sipley J, Nagase H, French DL, Quigley JP. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. J Biol Chem 1999; 274 (19): 13066-76.
74. Lee SL, Dickson RB, Lin CY. Activation of hepatocyte growth factor and urokinase/plasminogen activator by matriptase, an epithelial membrane serine protease. J Biol Chem 2000; 275 (47): 36720-5.
75. Takeuchi T, Harris JL, Huang W, Yan KW, Coughlin SR, Craik CS. Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. J Biol Chem 2000; 275 (34): 26333-42.
76. Monsky WL, Lin CY, Aoyama A, Kelly T, Akiyama SK, Mueller SC et al. A potential marker protease of invasiveness, seprase, is localized on invadopodia of human malignant melanoma cells. Cancer Res 1994; 54 (21): 5702-10.
77. Gonzalez-Gronow M, Grenett HE, Weber MR, Gawdi G, Pizzo SV. Interaction of plasminogen with dipeptidyl peptidase IV initiates a signal transduction mechanism which regulates expression of matrix metalloproteinase-9 by prostate cancer cells. Biochem J 2001; 355 (Pt 2): 397-407.
78. Afar DE, Vivanco I, Hubert RS, Kuo J, Chen E, Saffran DC et al. Catalytic cleavage of the androgen-regulated TMPRSS2 protease results in its secretion by prostate and prostate cancer epithelia. Cancer Res 2001; 61 (4): 1686-92.