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INORGANIC PYROPHOSPHATE (PP_I) IN PATHOLOGIC CALCIFICATION OF ARTICULAR CARTILAGE

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1. ABSTRACT

Physiologic levels of extracellular PP_i, which suppresses hydroxyapatite crystal growth, must be maintained by articular chondrocytes and resident cells in many othee tissues in order to prevent pathologic calcification. However, extracellular PP_i rises in articular cartilage in direct association with aging. Matrix supersaturation with PP_i stimulates chondrocalcinosis manifesting as calcium pyrophosphate dihydrate (CPPD) crystal deposition. Extracellular PP_i levels are normally held in check by balances in PP_i generation by nucleotide pyrophosphatase phosphodiesterase (NPP/NTPPPH) activity relative to PP_i degradation by pyrophosphatases, by balance effects of cytokines and growth factors, and by transport of PP_i from the cell interior involving the multiple-pass transmembrane protein ANK. But these mechanisms become dysrgulated in aging and osteoarthritic (OA) cartilage and extracellular PP_i excess supervenes, mediated in large part by upregulated NPP1 and ANK expression in articular cartilage. Conversely, NPP1 and ANK deficiency states were recently linked to phenotypically similar forms of spontaneous soft tissue calcification with hydroxyapatite (HA). Here, we focus on recent advances in understanding of PP_i metabolism and NPP1 and ANK function pertinent to the pathogenesis of pathologi matrix calcification in articular cartilage.

2. INTRODUCTION

Supersaturation of certain solutes in body fluids may culminate in the extracellular deposition of distinct crystals and calculi and in dystrophic or organized matrix calcification. Pathologic calcification can manifest in the extracellular matrix of certain connective tissues such as the artery wall in subjects with atherosclerosis or chronic renal failure. Significantly, articular cartilage, unlike growth plate cartilage, is specialized to avoid the process of matrix calcification. But the matrix of articular hyaline cartilage, like that of fibrocartilaginous menisci, lends itself well to pathologic calcification, particularly in association with certain changes in matrix composition in aging and OA (1, 2).

PP_i is a potent inhibitor of the nucleation and propagation of basic calcium phosphate crystals such as HA (3). Concordantly, maintenance of physiologic extracellular PP_i levels by chondrocytes and certain other cells serves to suppress calcification with HA, as illustrated in certain mouse models of deficient PP_i generation and transport, as well as a variant of human infantile arterial calcification associated with periarticular calcification discussed below. But the relatively unique capacity of chondrocytes to produce copious amounts of extracellular PP_i is double edged, as it is a major factor in promoting CPPD crystal deposition (Figure 1). Furthermore, excess PP_i generation can promote HA crystal deposition by providing a source for increased P_i generation via PP_i hydrolysis by inorganic pyrophosphatase and alkaline phosphatase activities (Figure 1). Indeed, depending on cartilage ATP and PP_i concentrations, and the level of activity of P_i-generating ATPases and pyrophosphatases, CPPD and HA crystal formation may be jointly promoted in cartilage, an event that commonly occurs clinically in OA cartilage (1), as discussed further below.

3. PP_i GENERATION AND DEGRADATION

PP_i is comprised of two inorganic phosphate (P_i) molecules linked by a hydrolyzable high energy ester bond (Figure 2). Widely used in drug therapy for mineralization disorders is the class of synthetic, therapeutic compounds

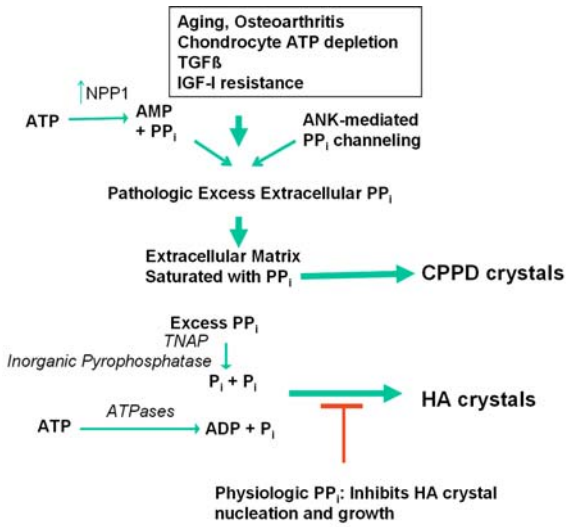

Figure 1. Proposed PP_i-dependent mechanisms stimulating CPPD and HA crystal deposition in aging and osteoarthritis (OA): Roles of ATP and PP_i Metabolism and inorganic phosphate (P_i) generation in pathologic cartilage calcification. This model presents mechanisms underlying the common association of extracellular PP_i excess with both CPPD and HA crystal deposition in OA and chondrocalcinosis cartilages, as well as the paradoxical association of extracellular PP_i deficiency (from defective ANK or PC-1/NPP1 expression) with pathologic calcification of articular cartilage with HA crystals *in vivo*. Factors driving pathologic calcification are indicated in green and physiologic factors suppressing calcification in red. Excess PP_i generation in aging cartilages in idiopathic CPPD deposition disease of aging, and in OA cartilages, is mediated in part by marked increases in nucleoside triphosphate pyrophosphohydrolase (NTPPPH) activity, mediated in large part by the PC-1/NPP1 isoenzyme. In idiopathic chondrocalcinosis of aging and in OA, there are substantial increases in joint fluid PP_i derived largely from cartilage. NPP1 not only directly induces elevated PP_i but also matrix calcification by chondrocytes *in vitro*. Depending on extracellular availability of substrate PP_i and the activity of pyrophosphatases, the availability of substrate ATP and the activity of ATPases, and other factors such as substantial local Mg^{++} concentrations, HA crystal deposition, as opposed to CPPD deposition, may be stimulated. In this model, excess extracellular PP_i also may result from heightened release of intracellular PP_i via increased ANK expression in OA and abnormal ANK function in familial chondrocalcinosis, as well as from deficient activity of pyrophosphatases (such as tissue-nonspecific alkaline phosphatase (TNAP) and possibly inorganic pyrophosphatase) in certain primary metabolic disorders. Also illustrated at the top of this schematic is the role in cartilage calcification in OA and aging of altered TGFβ expression and responsiveness, which drives PP_i generation and release mediated via NPP1 and ANK, and diminished responsiveness to IGF-I, which normally suppresses elevation of chondrocyte extracellular PP_i.

termed bisphosphonates (Figure 2), have a carbon instead of an oxygen molecule separating two phosphate, thereby allowing these drugs to serve in part as nonhydrolyzable PP_i analogues. Natural compounds similar in structure to PP_i include the calcific crystallization inhibitor phosphocitrate (4) (Figure 2). Phosphocitrate is a synthetic product of citrate and ATP that is highly enriched in mitochondria. But some of the described effects of phosphocitrate on calcification may be mediated in part by citrate effects, and this review focuses on PP_i.

PP_i generation and disposal are finely balamced (3). Though a PP_i synthase exists in primitive organisms, PP_i does not appear to be synthesized *de novo* in mammalian cells, where PP_i is formed either as a metabolic byproduct of numerous biochemical and biosynthetic reactions (3) or directly by pyrophosphohydrolysis of the phosphodiesterase I bond in purine and pyrimidine nucleoside triphosphates (NTPPPH activity) (EC 3.6.1.8) (5). NTPPPH activity is a primary effect of two members of the Nucleotide Pyrophosphatase Phosphodiesterase (NPP) family (5, 6), PC-1/NPP1 and B10/NPP3 (3, 7, 8). These ~130 kDa transmembrane ecto-enzymes form disulfide-bonded homodimers (5, 6). Secreted NPP1 is generated at the plasma membrane via proteolysis and is the predominant circulating NPP1 in plasma (5, 9).

In plasma, normal PP_i concentrations are in the micromolar range, and urinary PP_i excretion is substantial (3). Much of circulating PP_i is believed to be released from the liver and taken up by bone (3). The extent and functional significance of PP_i binding to plasma proteins are undefined.

Analogous to effects of not only inorganic phosphate (P_i) (10, 11) but also bisphosphonates (12, 13), intracellular and extracellular PP_i appears to directly influence gene expression and cell functions, as discussed below. Hence, it is not surprising that specialized mechanisms exist for both PP_i transport and PP_i degradation, as well as generation of PP_i by NTPPPH activity. Mammalian extramitochondrial mechanisms for PP_i production, degradation, and transport were recently reviewed in depth (3). These mechanisms include PP_i transport by ANK discussed below. They also include the essential action in mineralization of tissue-nonspecific alkaline phosphatase (TNAP), an ecto-enzyme that generates pro-mineralizing P_i by not only ATPase activity but also by catalyzing PP_i degradation at an alkaline pH optimum, exerting major effects on extracellular PP concentrations (14-16). Recent comparative analyses of primary structures have revealed that alkaline phosphatases and the PDNP family of NTPPPH metalloenzymes share certain features that mediate enzyme activity (17, 18) It appears that a substantial fraction of intracellular PP_i is generated in the mitochondria and intracellular and extracellular PP_i concentrations are both under the regulation of mitochondrial energy metabolism (3, 19).

As reviewed recently (3), mitochondria carry out a complete catalytic cycle employing P_i that results in the spontaneous synthesis of ATP and PP_i . Intramitochondrial PP_i also is under the control of calcium, a suppressor of mitochondrial matrix inorganic pyrophosphatase activity.

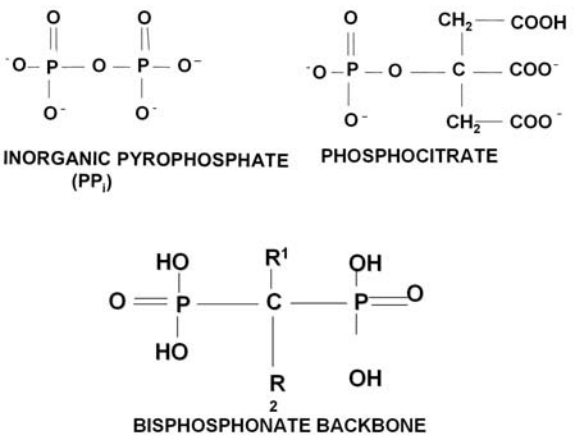

Figure 2. Structure of PP_i relative to two analogues of PP_i (phosphocitrate and bisphosphonates). Structure of PP_I is illustrated on the upper left, whereas the upper right depicts the structure of phosphocitrate, a naturally occurring phosphorylated polycarboxylic acid that is particularly abundant in mitochondria. Phosphocitrate also is released within the renal tubules and detectable in the urine. The structural framework for synthetic bisphosphonates is illustrated in the bottom panel, where the nature of the R2 side chain determines whether the agents are aminobisphosphonates or nonaminobisphosphonates, and modifies their functional properties. PP_I, phosphocitrate, and bisphosphonates act not only to modulate crystallization inhibitor of hydroxyapatite (HA) but have other effects on cell function, as discussed in the text and reviewed elsewhere for bisphoshonates. For example, simple bisphosphonates that closely resemble PP_i (e.g., clodronate, etidronate) can be metabolically incorporated into non-hydrolyzable ATP that accumulate intracellularly and modulate apoptosis. More potent, nitrogen-containing bisphosphonates (e.g., pamidronate, alendronate, risedronate, zoledronate) can act as analogues of isoprenoid diphosphate lipids, and thereby affect mevalonate pathway activity and can regulate activity of small GTP-binding proteins such as ras.

We established that mitochondrially-derived ATP is a major substrate for NTPPPH-mediated generation of both intracellular and extracellular PP_i in the chondrocyte (19). NTPPPH ecto-enzyme-mediated PP_i synthesis also takes place in the lumen of the endoplasmic reticulum, and likely also in the Golgi, where an active transport system exists to pump in ATP, as reviewed (3).

Extracellular PP_i has been extensively studied in a pathophysiologic context in mammals with respect to inhibitory effects on hydroxyapatite nucleation and crystal growth (3). In lower organisms, PP_i (like ATP) serves as a primordial "high-energy" compound, and PP_i (or polyphosphates) are able to substitute for ATP under certain circumstances, such as in glycolysis-related reactions in harsh environments under conditions where respiration is attenuated (3). But functional effects of free intracellular PP_i are not well understood in higher organisms and are likely underestimated. PP_i can interact with and potentially modify the activity of certain ATPases and can modify iron transport, mitochondrial metabolism, protein phosphorylation, DNA replication, protein synthesis, and calcium release from mitochondrial stores (reviewed in reference 3).

4. PP_i METABOLISM IN ARTICULAR CARTILAGE

In the pericellular matrix of aging and osteoarthritic (OA) cartilages, changes in chondrocyte differentiation and viability, alterations in matrix composition, and a dysregulated increase in chondrocyte extracellular PP_i promote deposition of CPPD and/or basic calcium phosphate crystals including HA (1, 2, 20) (Figure 1). Cartilage crystal deposition and crystal trafficking to the synovial membrane are believed to worsen the course of degenerative joint disease by promoting expression of inflammatory and cartilage-matrix-degrading genes by synovial lining cells, resident mononuclear phagocytes and chondrocytes, and by triggering inflammatory synovitis (21).

As cited above, physiologic levels of extracellular PP_i must be maintained by chondrocytes to suppress matrix deposition of HA (Figure 1), apparently reflecting antagonistic effects of PP_i and P_i on mineralization. The balance between PP_i and P_i in mineralizing tissues is complex partly because extracellular PP_i is a major source of P_i generated by TNAP activity, and P_i clearly promotes mineralization (14-16). Cytokine and growth factor balance also appear to keep extracellular PP_i levels in check. For example, in normal chondrocytes, TGFβ increases and IGF-I decreases extracellular PP_i (1, 22). IGF-I also inhibits the capacity of TGFβ to induce elevated extracellular PP_i (22, 23). The interplay between TGFβ and IGF-I on PP_i metabolism is partly mediated by expression of the matrix protein Cartilage Intermediate Layer Protein-1 (CILP), an IGF-I inhibitor induced by TGFβ (23). Significantly, IL-1 and TNFα also decrease extracellular PPi and inhibit the capacity of TGFβ to induce elevated extracellular PP_i (24).

Extracellular PP_i rises markedly in cartilage in direct association with aging and OA, and resultant matrix supersaturation with PP_i can stimulate both CPPD and HA deposition (reviewed in 1, 3, 7). Physiologic chondrocyte PP_i metabolism depends largely on the balance between PP_i generation and degradation, on a normal capacity for PP_i transport, and on normal chondrocyte responsiveness to growth factors. Interruption in these regulatory checks and balances appears to occur in aging and OA, under the influence of altered chondrocyte responsiveness to growth factors.

5. PP_i GENERATION BY CHONDROCYTES

Chondrocytes produce PP_i by a variety of biosynthetic reactions and by the action of the aforementioned NPP family isoenzymes that hydrolyze nucleoside triphosphates at the phosphodiester I bond by NTPPPH activity (7, 8). Three NPP family ecto-enzymes with NTPPPH activity (NPP1-3) share disulfide-bonded

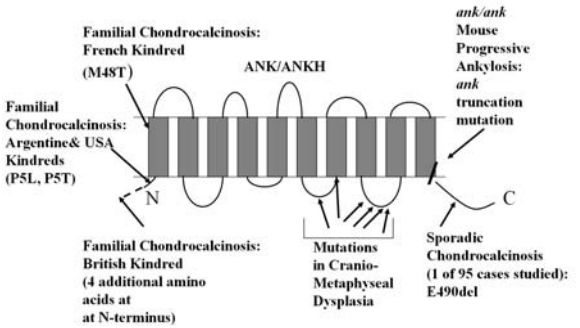

Figure 3. Model for Multiple-Pass Membrane Protein Structure of ANK (human ANKH gene product) and for ANK Mutations Associated with Distinct Skeletal Phenotypes. The Figure schematizes the putative multiple-pass transmembrane protein structure of ANK/ANKH, which appears to function in PP_i channeling from the cytosol to the extracellular space. Distinct mutations in ANK promote distinct phenotypes. As depicted here, impairment of ANK function via homozygosity for a C-terminal cytosolic domain *ank* truncation mutation in murine progressive ankylosis *ank/ank* mice leads to a decrease in extracellular PP_I, resulting in deposition of HA crystals in articular cartilage, OA, and peripheral synovial and intervertebral bony ankyloses. NPP1 deficient mice (*ttw/ttw* mice and NPP1-/- mice) have a remarkably similar phenotype to that of *ank/ank* mice, linked by decreased extracellular PP_i. The Figure also summarizes sites of known ANKH gene mutations associated with autosomal dominant chromosome 5p-linked familial chondrocalcinosis (CPPD crystal deposition disease) and with sporadic chondrocalcinosis in 1 subject. Some but not all the described ANKH mutations linked to human chondrocalcinosis promote chronic low-grade extracellular PP_i excess resulting in CPPD crystal formation. The sites of autosomal dominant ANKH gene mutations implicated in craniometaphyseal dysplasia (CMD) also are depicted. CMD is characterized by progressive thickening and increased mineral density of craniofacial bones and hyperostotic flaring at metaphyses in long bones, as well as the classic "mask" facies. The effects of the locations of ANKH mutations on PP_i transport in CMD are currently unknown.

homodimeric structures as exemplified by NPP1 (5, 6). But up to half of extracellular PP_i in skeletal cells and fibroblasts is generated by the activity of NPP1 (9, 25), and NPP2 appears to act primarily as a lysophospholipase D (25a) and T-type ATPase rather than PP_i-generating NTPPPH, whereas NPP3 appears to principally influence intracellular PP_i in chondrocytes (7).

Osteoblasts and chondrocytes have particularly high levels of NTPPPH specific activity. Moreover, chondrocyte NTPPPH activity increases in direct condordance with cartilage PP_i generation (to an average of double normal levels) in a donor age-dependent manner (3, 26), as illustrated again recently in both human knee meniscal cells (3, 7). The age-dependent increases in NTPPPH activity are directly linked to chondrocalcinosis (26). Synovial fluid levels of the NTPPPH substrate ATP also rise to approximately double control levels in idiopathic CPPD crystal deposition disease of aging (27). Furthermore, up-regulation of NPP1 but not the other NTPPPH isoenzymes is associated with crystal deposition by chondrocytic cells *in situ* and *in vitro* (7).

The ability of certain cartilage-expressed factors to modulate chondrocyte extracellular PP_i levels is transduced in part by changes in NTPPPH activity and the availability to NTPPPH of substrate ATP (3, 27). In this regard, TGFβ increases cartilage NTPPPH activity and extracellular PP_i levels in a manner that directly correlates with donor age (7, 28). The TGFβ-stimulated cellular program for chondrocyte extracellular PP_i elevation includes substantial increases in ATP generation (29), increased transcription of NPP1 (24) and stimulation of NPP1 movement to the plasma membrane (where NPP1 remains an ecto-enzyme) (8), in a manner dependent on the cytosolic tail dileucine motif of NPP1 (30). The ability of IL-1 and TNF□ to suppress extracellular PP_i correlates with suppression of NPP1 expression and NTPPPH specific activity in chondrocytes (24). The mechanism by which IGF-I suppresses extracellular PP_i may involve suppression of expression of the PP_i transporter ANK (31) discussed below.

6. ANK, NPP1, AND PP_i METABOLISM IN MATRIX CALCIFICATION

Osteoblasts, chondrocytes, and certain other cells release intracellular PP_i by a membrane transport system involving ANK, a 54 kDa polypeptide with a predicted multiple-pass transmembrane configuration (32). ANK expression is up-regulated in OA and chondrocalcinotic cartilages (31, 33). A truncation mutation of the C-terminal cytosolic domain, the last of six putative cytosolic domains in ANK, occurs naturally in *ank/ank* mice, and this mutation is associated with elevation of intracellular PP_i and a decrease in extracellular PP_i (25, 32, 33). Wild type ANK decreases intracellular PP_i and elevates extracellular PP_i concentrations in normal cells (32, 33). The *ank* mutant lacking the C-terminal (sixth) cytosolic domain appears to have totally incapacitated ANK PP_i transport activity (32).

ANK and NPP1 appear to co-dependently function to raise extracellular PP_i (25), suggesting that ANK moves the specific fraction of intracellular PP_i generated by NPP1. Consistent with this model is the remarkable similarity in the consequences of deficient ANK and PC-1 function *in vivo*. Both *ank/ank* mice and NPP1 deficient mice develop a progressive matrix hypermineralizing phenotype that with increasing age comes to include OA and extensive articular cartilage HA deposits, spinal ligament hyperostosis, arterial calcification, and ossific fusion of peripheral joints (15, 16, 25, 32, 34). Deficiency of NPP1 also produces certain comparable phenotypic features in humans (9) and is the predominant cause of human arterial calcification of infancy, a frequently lethal disease characterized by widespread artery media calcification and common periarticular HA deposits (35, 36). Mutations widely spread through the NPP1 extracellular domain can impair catalytic activity (35, 36).

Unlike cultured cells of *ank/ank* mice, NPP1-deficient cells demonstrate low intracellular as well as extracellular PP_i levels (25). Thus, the common basis for the remarkably similar hypermineralizing phenotypes seen in *ank/ank* mice and in NPP1 null mice (and the hypercalcification seen in human NPP1 deficiency) clearly rests in depression of extracellular PP_i.

7. NPP1 AND MATRIX VESICLES (MVS) IN CALCIFICATION

Chondrocyte-derived and osteoblast-derived membrane-limited secretory bodies termed matrix vesicles (MVs) have the capacity to provide a sheltered environment for initiation of HA and CPPD crystal formation in a manner modulated by the concentration of PP_i and other constituents associated with MVs (37). NPP1 is clearly the principal NTPPPH associated chondrocyte-derived and osteoblast-derived MVs, as confirmed recently by analyses of NPP1-/- mouse cells (30). Significantly, chondrocyte differentiation and a variety of calciotropic hormones and cytokines (including 1,25 dihydroxyvitamin D3, TGFβ and IL-1) can regulate the NPP1 content, NTPPPH and alkaline phosphatase activities, PP_i content, and other compositional features of MVs (37-39). We have not seen concentrated ANK localization in MVs (16).

Chondrocyte maturation to hypertrophy is associated with heightened PP_i generation and shedding of mineralization-competent matrix vesicles (MVs) (38). Chondrocytic MVs, like osteoblast-derived MVs are enriched markedly in NPP1 (7, 14, 15, 30, 40) as well as TNAP relative to the plasma membrane, and the catalytic domains of NPP1 and TNAP are predominantly exposed at the external face of MVs. NPP1 and TNAP exert critically important, mutually anatagonistic regulatory effects on HA deposition *in vivo*, exerted partly at the level of the MV (14, 15). In contrast, TNAP and B10/NPP3, which is predominantly localized intracellularly in mineralizing cells, do not appear to be mutually antagonistic in PP_i metabolism (14). As such, the mechanisms underlying co-localization of TNAP and NPP1 at the plasma membrane and in MVs are of substantial interest.

Excess NPP1 expression, but not excess NPP2 or NPP3 expression, was associated with increased meniscal cell apoptosis (presumably mediated by PP_i excess) (40). Concurrently, we observed the release of functionally altered MVs in chondrocytes expressing excess NPP1 (40). Specifically, such MVs, which presumably were enriched in apoptotic bodies, contained increased amounts of the calcium-binding protein annexin V and precipitated more calcium in both an ATP-dependent and ATP-independent manner (40).

8. EFFECTS OF PP_i ON OSTEOPONTIN EXPRESSION

Recently, it has been recognized that P_i regulates the expression of certain genes in skeletal cells (41). These effects include stimulation by P_i (mediated by plasma membrane sodium-phosphate co-transport) of expression of osteopontin, an inhibitor of HA crystal deposition and promoter of mineral resorption (41). Significantly, exogenous PP_i promotes osteopontin and MMP-13 expression (16, 25, 33). Hence, altered PP_i generation by skeletal cells likely modulates chondrocyte differentiation partly by regulating gene expression.

We observed that a deficiency state for extracellular PP_i in NPP1-/- and *ank/ank* mice was associated with deficient osteopontin expression, and osteopontin deficiency was a critical effect facilititating increased calcification *in vitro* (25). This represesents the remarkable case of a deficiency of one HA crystal growth inhibitor inducing a deficiency of a second HA crystal growth inhibitor. Because osteopontin knockout mice have only mild changes in bone mineral crystal size, as opposed to the marked phenotypic abnormalities in extracellular PP_i-deficient mice, PP_i clearly is more elevated than osteopontin in the physiologic hierarachy of HA crystal growth inhibitors.

9. MODEL FOR DYSREGULATED PP_i METABOLISM IN AGING AND OA ARTICULAR CARTILAGES

We have demonstrated that chondrocyte mitochondrial dysfunction associated with spontaneous Hartley guinea pig knee OA promtes ATP depletion, and that increased NTPPPH activity and extracellular PP_i develop concurrent with the ATP-depleted state (19). Hence, increased ATP-scavenging by energy-depleted chondrocytes likely drives extracellular PPi excess in OA and aging chondrocalcinotic cartilages. In addition, in the model presented in Figure 1, PP_i metabolism becomes dysregulated in part via increased PP_i generation in response to TGFβ (8), mediated in large part by up-regulation of ANK and NPP1 and their synergistic effects. In this model, PP_i metabolism becomes dysregulated in aging cartilage in part via lessening of the inhibitory effect of IGF-I on extracellular PP_i. Articular chondrocytes in aging cartilages demonstrate altered IGF-I responsiveness (42), and in OA, the ability of IGF-I to stimulate and maintain normal chondrocyte differentiation appears to be diminished (43). The IGF-I hyporesponsiveness in OA appears to be multifactorial (42, 44). Increased levels of the IGFBPs 2, 3, 4, and altered IGFBP complex formation with IGF-I and the acid-labile subunit (ALS) (45) could play a role, as IGF-I receptor levels do not decrease in OA chondrocytes (46). The capacity of NO to suppress ligand-induced phosphorylation of the IGF-I receptor (44) and to inhibit proteoglycans synthesis (47) also may be significant modulators of IGF-I action in OA. It appears that one of the consequences of increased CILP-1 expression in aging cartilage is interference with the regulatory effects of IGF-I on PP_i metabolism, thereby promoting increased extracellular PP_i and CPPD crystal deposition.

10. DYSREGULATED ARTICULAR PP_i METABOLISM AND CPPD CRYSTAL DEPOSITION DISEASE IN PRIMARY METABOLIC DISORDERS

Hypophosphatasia due to TNAP deficiency

states, hypomagnesemic conditions (including the Gitelman's variant of Bartter's Syndrome), hemochromatosis, and hyperparathyroidism are linked to secondary CPPD crystal deposition disease (48). Increased joint fluid PP_i levels in each of these conditions indicates a shared theme of cartilage PP_i excess (49), likely mediated by effects of magnesium, iron, copper, and calcium on NPP1 catalytic activity and on pyrophosphatase activity.

11. ANKH MUTATIONS IN FAMILIAL CPPD CRYSTAL DEPOSITION DISEASE

Familial chondrocalcinosis is clinically heterogeneous (50). Two major chromosomal linkages, 8q and 5p, have been identified in studies of familial CPPD deposition disease (50). CCAL1 is the designation for the linkage with chromosome 8q of both early-onset OA and chondrocalcinosis in a New England family (51). Chromosome 5p-linked chondrocalcinosis (CCAL2) is broadly distributed and has been studied in greater detail than 8q chondrocalcinosis (52-57, 57a), with linkage to the ANKH gene now well-established. A search for ANKH gene mutation in 95 subjects with sporadic chondrocalcinosis also uncovered a unique mutation in one subject (56). A subset of ~4% of severe human "idiopathic" chondrocalcinosis of aging has recently been linked to homozygosity for an ANKH promoter mutation that promotes increased ANKH expression (58).

Modeling of the PP_i channeling function of human ANK has posited 10 or 12 membrane-spanning domains in ANK with an alternating inside/out orientation (32) and with a central channel to accommodate the passage of PP_i (59) (Figure 3). Mutations at different locations in mouse ANK and in ANKH can affect function and the skeleton in a manner including autosomal dominant chondrocalcinosis (56, 57), the phenotype of *ank/ank* mice (32), and other abnormalities (59, 60) (Figure 3). Clinical heterogeneity even for chondrocalcinosis associated with human ANK mutations (56, 57) suggests differing functional effects of ANK mediated by specific regions of the molecule.

In one form of 5p familial chondrocalcinosis, subtle "gain of function" of intrinsic ANKH PP_i channeling activity, putatively via extension of the N-terminal domain by 4 amino acids, appears to lead to chronic, low-grade chondrocyte "PP_i leakiness", thereby putatively causing matrix supersaturation with PP_I, CPPD crystal deposition (56, 57). However, not all ANKH mutants linked to familial chondrocalcinos directly raise extracellular PP_i (58). For example, marked intracellular PP_i elevation was described in cell lines from the French chromosome 5p familial chondrocalcinosis kindred (61, 62). With respect to chondrocalcinosis in OA and aging, ANKH expression is highly regulated, and secondary alterations in chondrocyte expression of not only wild type ANKH but also NPP1 likely drive PP_i supersaturation in cartilage in idiopathic/sporadic and OA-associated CPPD crystal deposition disease.

Last, mutations clustered in the fifth cytosolic domain of the human ANK homologue ANKH encoding for single amino acid deletions or nonconservative amino acid substitutions were recently linked to a human autosomal dominant disease, Craniometaphyseal Dysplasia (CMD), in multiple families (59, 60) (Figure 3). CMD features progressive thickening and increased mineral density of craniofacial bones ("mask facies") and abnormally developed (i.e., hypertrophic and flared) metaphyses of long bones (59, 60). A theoretical model has been proposed in which enhanced PP_i "leakiness" through the central pore of the channel is a factor in CMD pathogenesis (59). However, there is no proof of dysregulated PP_i metabolism in CMD and the disease may reflect abnormalities in bone resorption related to an undefined abnormality in ANKH function or an aberrant locus for ANKH PP_i transport function.

12. PERSPECTIVE

Studies of extracellular PP_i deficiency states in humans and mice have revealed the profound physiologic inhibitory role of PP_i in extracellular matrix calcification in articular cartilage, spinal ligaments, synovium, and arteries. Since the discovery more than 3 decades ago of severalfold increased joint fluid PP_i in OA and even more markedly increased joint fluid PP_i in chondrocalcinosis (63), and more than 20 years removed from the first observations that normal chondrocytes robustly produce extracellular PP_i (64), it also has become clear that PP_i functions beyond a substrate regulating matrix deposition of HA and CPPD crystals. Toxic effects of extracellular PP_i on chondrocyte viability and the capacity of PP_i to modulate gene expression and differentiation indicate far-reaching effects of PP_i on cell function that modulate physiologic and pathologic calcification, and underscore the appropriateness of the term "pyrophosphate arthropathy" to describe CPPD-associated chondrocalcinosis. Last, molecular characterization of effects of ANK/ANKH and NPP1 on mineralization and specific genetic linkages of ANKH and NPP1 with human diseases have provided rational targets for novel therapeutics for specific calcification disorders.

13. ACKNOWLEDGEMENTS

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