Commentary

The *ank* gene story

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Abstract

The underlying molecular defect resulting in the abnormal calcification observed in *ank/ank* mice has been identified. The responsible nonsense mutation affects the protein product of *ank,* resulting in diminished production of extracellular inorganic pyrophosphate, an important inhibitor of nucleation and of the growth of apatite crystals. The *ank* gene product is one of several cell membrane proteins, including ectonucleoside triphosphate pyrophosphohydrolase enzymes and alkaline phosphatase, that regulate extracellular inorganic pyrophosphate levels and thereby regulate mineralization.

Keywords: *ank/ank* mice, apatite, basic calcium phosphate, calcium pyrophosphate dihydrate, inorganic pyrophosphate, pathologic mineralization

Introduction

A landmark investigation into the genetic basis of murine progressive ankylosis has clarified the physiologic role of extracellular inorganic pyrophosphate (ePPi) in suppressing pathologic deposition of basic calcium phosphate (BCP) (an inclusive term for hydroxyapatite, octacalcium phosphate, and tricalcium phosphate) in articular tissue [1]. A cell membrane protein, ANK, affects ePPi concentrations and the balance of mineralization in articular tissues.

Excess ePPi promotes pathologic mineralization with CPPD crystals

Excess accumulation of ePPi has long been recognized as an important factor in the mineralization of cartilage with calcium pyrophosphate dihydrate (CPPD) crystals. Elevated levels of ePPi are routinely noted in synovial fluids of patients with CPPD deposition disease [2,3]. Chondrocytes are the likely source of the ePPi that participates in the formation of these crystals [4,5]. The elaboration of ePPi by chondrocytes is a bioregulable process, enhanced by transforming growth factor β, ascorbate, retinoic acid, bone morphogenetic protein, transglutaminase, and thyroid hormones and diminished by parathyroid-hormone-related peptide isoforms, insulin-like growth factor-1, tumor necrosis factor α, and interleukin 1. Porcine chondrocytes from aged donors make more ePPi than do chondrocytes from young donors [6]. Signaling mechanisms involved in the regulation of ePPi formation are poorly understood, but adenylyl cyclase activation decreases and protein kinase C activation increases the accumulation of ePPi in media surrounding cartilage or chondrocyte cultures [7]. In addition to causing CPPD crystal formation, excess ePPi accumulation may also affect BCP mineralization.

CPPD crystal deposits and elevated ePPi levels are particularly prominent in adult hypophosphatasia, congenital deficiency of tissue-nonspecific alkaline phosphatase. In hypophosphatasia, the predominant phenotypic disease expressions are rickets and osteomalacia. Murine models of hypophosphatasia indicate that the nucleation and initial growth of BCP crystals within matrix vesicles of mineralizing

ABC = ATP binding cassette; BCP = basic calcium phosphate; CPPD = calcium pyrophosphate dihydrate; ePPi = extracellular inorganic pyrophosphate; iPPi = intracellular PPi; NTPPPH = nucleoside triphosphate pyrophosphohydrolase.

bone are normal. However, with loss of vesicle integrity, further BCP crystal growth is suspended [8]. Possibly these BCP deposits are coated by ePPi, preventing further mineral accretion. These results suggest that the adsorption of PPi to BCP occurs *in vivo* as predicted by the *in vitro* studies of Fleisch and coworkers [9].

If excess ePPi inhibits the nucleation and growth of BCP, does deficiency of ePPi promote its formation?

Deficient ePPi promotes pathologic mineralization with BCP crystals

Tiptoe walking (*ttw*) mice have an underlying nonsense mutation in PC-1, a membrane-bound ectoenzyme that can generate ePPi from extracellular nucleoside triphosphates [10]. This enzyme has been extensively studied by Terkeltaub and coworkers, who found that it has an important role in maintaining levels of extracellular and perhaps intracellular PPi [11,12]. Phenotypically, *ttw* mice develop excess calcification of the ligaments of the axial skeleton, resulting in myelopathy and an abnormal gait. However, despite the putative role of PC-1 in ePPi generation, reduced ePPi formation and accumulation have not yet been directly demonstrated in this animal model. (Interestingly, mutated PC-1 and decreased ePPi levels have been identified in a patient presenting with severe periarticular and vascular calcification [13].) The recently published *ank* gene story establishes the direct tie between low ePPi levels and excess BCP mineralization of articular structures in another animal model.

Murine progressive ankylosis is the result of an autosomal recessive mutation in *ank*, producing spontaneous ankylosis of peripheral and axial joints with bony, BCP-containing tissue [14]. Disease progression in mutant *ank/ank* mice can be halted by treatment with phosphocitrate, a compound that adsorbs to BCP crystals, as does PPi [15]. Ho, Johnson, and Kingsley have identified the mutation responsible for murine progressive ankylosis and linked it directly to abnormally suppressed cellular elaboration of ePPi [1]. These investigators from the Department of Developmental Biology and Howard Hughes Medical Institute at Stanford University School of Medicine performed genetic and physical mapping of the *ank* locus in the proximal mouse chromosome 15. Bacterial artificial clones derived from wild-type mice were tested to determine whether they could rescue *ank* mutant phenotypes in transgenic mice. Remarkably, the mice transgenic for wild-type *ank* developed neither the joint stiffness nor the joint BCP deposits characteristic of *ank/ank* mutants, indicating that the mutated gene product played a direct role in the excess mineralization.

The *ank* mutation was found to be a nonsense G-to-T substitution. The predicted *ank* product, termed ANK, is a 54-kD protein with numerous hydrophobic stretches, glycosylation sites, and phosphorylation sites. Immunofluorescence studies show that it is localized mainly in the cell membrane. Northern blot analysis and *in situ* hybridization studies revealed *ank* mRNA in multiple adult tissues and in developing articular cartilage. Fibroblasts from *ank/ank* mutants contained excess intracellular PPi (iPPi) and made little ePPi in comparison with fibroblasts from wild-type mice. Overproduction of ANK in mutant *ank/ank* mouse fibroblasts reversed the alterations in ePPi and iPPi levels, indicating a crucial role for ANK protein in controlling PPi localization. The effect of ANK protein was blocked by probenecid. This weak organic anion inhibits transmembrane anion transport and has been implicated in decreasing ePPi elaboration by articular chondrocytes [16]. Its effect suggests that ANK may function as or regulate an anion channel.

Unresolved questions

Many questions remain unanswered concerning the role of ANK in regulating ePPi metabolism.

Is ANK a channel through which PPi can traverse the plasma membrane or does it regulate flow through an adjacent channel? Either a role as a channel or a regulatory function is consistent with the published data. How does ANK relate to ectoenzymes that generate ePPi? Substantial data suggest that nucleoside triphosphate pyrophosphohydrolases (NTPPPHs) such as PC-1 and cartilage intermediate layer protein play an important role in elaboration of chondrocyte ePPi. Levels of NTPPPH activity in joint fluids correlate directly with ePPi levels [17]. And reduction of substrate (ATP) or ectoenzyme activity (by trypsinization) markedly reduces ePPi formation [18,19]. Conversely, supplying exogenous substrate and overexpression of the PC-1 form of NTPPPH enhance ePPi formation [11,20]. Providing exogenous ATP to cartilage leads to not only increased ePPi concentrations but also actual formation of CPPD crystals [21]. Perhaps ANK serves as a conduit (or regulates such a channel) for ATP, which is then converted extracellularly to ePPi by ecto-NTPPPH. The probenecid data may reflect its ability to influence ATP binding cassette (ABC) transporters, potential facilitators of ATP egress, rather than its effect on anion channels [22]. How can ANK explain the concurrence of BCP and CPPD crystals, so often seen together in joint fluids? In some studies, the two occur concomitantly more often than either occurs alone [17]. It would seem that BCP should be a result of deficient ANK expression and CPPD, of excess ANK expression. The presence of the two together is unexplained. However, it is noteworthy that articular cartilage vesicles capable of generating both species of calcium-containing crystals have been identified in cartilage, are enriched in NTPPPH activity, and are inhibited by phosphocitrate [23–25]. Does ANK reside in these membrane-derived structures?

Perhaps the most important result of these studies is heightened realization of the daunting task facing investi-

gators hoping to prevent the cartilage degeneration so often occurring in patients with diseases marked by the deposition of calcium-containing crystals. CPPD and/or BCP crystals are observed in 60% of joint effusions in patients with osteoarthritis [26]. Both have profound *in vitro* effects on synovial lining cells, including mitogenesis, synthesis and secretion of metalloproteinases, diminution of secretion of inhibitors of proteases, and release of cytokines and prostaglandins [27]. Theoretically, prevention of crystal formation would have a salutary effect on the degenerative process. Yet we now know that too much ePPi is undesirable (CPPD forms) and too little is equally unwanted (BCP forms). Prevention of the degenerative results of crystal deposition may depend upon maintaining ePPi concentrations within a narrow physiologic range. This contrasts with another disease of crystal deposition, gout, in which overcorrecting the hyperuricemia has no adverse consequences. A great deal more needs to be learned about the homeostasis of ePPi before fine-tuning of articular concentrations can be achieved.

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