Matrix Vesicles and Calcification

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Matrix vesicles (MVs) are extracellular, 100 nM in diameter, membrane-invested particles selectively located at sites of initial calcification in cartilage, bone, and predentin. The first crystals of apatitic bone mineral are formed within MVs close to the inner surfaces of their investing membranes. Matrix vesicle biogenesis occurs by polarized budding and pinching-off of vesicles from specific regions of the outer plasma membranes of differentiating growth plate chondrocytes, osteoblasts, and odontoblasts. Polarized release of MVs into selected areas of developing matrix determines the nonrandom distribution of calcification. Initiation of the first mineral crystals, within MVs (phase 1), is augmented by the activity of MV phosphatases (eg, alkaline phosphatase, adenosine triphosphatase and pyrophosphatase) plus calcium-binding molecules (eg, annexin I and phosphatidyl serine), all of which are concentrated in or near the MV membrane. Phase 2 of biologic mineralization begins with crystal release through the MV membrane, exposing preformed hydroxyapatite crystals to the extracellular fluid. The extracellular fluid normally contains sufficient Ca^{2+} and PO_4^{3-} to support continuous crystal proliferation, with preformed crystals serving as nuclei (templates) for the formation of new crystals by a process of homologous nucleation. In diseases such as osteoarthritis, crystal deposition arthritis, and atherosclerosis, MVs initiate pathologic calcification, which, in turn, augments disease progression.

Introduction

Biomineralization is under cellular control; that is, cells are required to generate a calcifiable matrix, and are responsible for initiating calcification at selected, nonrandom extracellular sites. After initiation, cells regulate progressive calcification by supplying sufficient extracellular Ca^{2+} and PO_4^{3-} plus other mineral supporting molecules, such as substrates for alkaline phosphatase [1]. Cells also control the rate and distribution of biomineralization through localized synthesis and secretion of mineral inhibiting molecules such as inorganic pyrophosphate (PPi) [2], proteoglycans [3], and matrix Gla protein [4].

Mineralization is initiated in cartilage, bone, and dentin in association with extracellular matrix vesicles (MVs) [5••]. MVs are approximately 100 nM in diameter, extracellular, membrane-invested particles that are generated by polarized budding and release from the surfaces of chondrocytes, osteoblasts, and odontoblasts [5••]. This process occurs as a temporal and spatial sequence in the growth plate of growing animals. Chondrocytes of the growth plate are arranged in columnar stacks, with proliferating cells in an upper zone overlying daughter chondrocytes. The latter undergo maturation (in the upper hypertrophic zone) and terminal differentiation and programmed cell death (apoptosis) in the lowest region of the growth plate [6]. A similar layering of less mature, superficial chondrocytes over more mature, deep-zone chondrocytes is seen in articular cartilage where MVs are more numerous in the deeper, tidemark area [7].

Maturation and differentiation of chondrocytes in the hypertrophic zone of growth plate is characterized by the expression of alkaline phosphatase [8] and the synthesis and secretion of type X collagen [9]. The tissue nonspecific isomer of alkaline phosphatase is localized by electron microscopic histochemistry at the outer surfaces of maturing hypertrophic chondrocytes and adjacent MVs of growth plate [8], where it is attached by a glycosyl linkage to phosphatidyl inositol of the cell and MV membranes [10]. Type X collagen is uniquely synthesized by hypertrophic chondrocytes, then secreted and incorporated into the lower hypertrophic and calcifying zone matrix [9].

The balance between chondrocyte proliferation and differentiation is regulated by a number of known and unknown interacting hormones, cytokines, and morphogenetic factors. The mechanism in which these factors interact to regulate growth plate cellular activities is just beginning to be understood. Factors that promote chondrocyte proliferation (thus inhibiting differentiation and MV biogenesis) include the following: parathyroid hormone–related peptide [11•], Bcl-2 protein [12], transforming growth factor [13], and fibroblast growth factor [14]. Factors that promote chondrocyte maturation and MV biogenesis include the following: thyroxine (T3) [15], retinoic acid [16], Indian hedgehog [17•], and bone morphogenetic proteins, especially bone morphogenetic protein-6 (BMP-6) [18•].

Matrix Vesicle Biogenesis

Matrix vesicles arise in skeletal tissues by budding and then pinching off from the outer cell membranes of chondrocytes, osteoblasts, and odontoblasts [5••]. The process of budding is polarized in all three of these cell types. Only a specific region of the outer cell membrane gives rise to MVs. With growth plate chondrocytes, MVs arise by blebbing from the lateral surfaces that lie adjacent to the vertically oriented longitudinal matrix septa [19]. In the growth plate, entry into differentiation by chondrocytes may be a prerequisite condition for MV biogenesis. Thus, factors known to promote chondrocyte differentiation, such as T3, retinoic acid, Indian hedgehog, or BMP-6, should theoretically be able to promote MV biogenesis. However, this hypothesis has not yet been adequately tested because of the unavailability of an experimental system in which the yield of mineralization-competent MVs generated under different experimental conditions can be quantified and correlated with the level of differentiation induced by the discussed factors. Recently, methods for primary culture of chick [20] and rat [21] growth plate chondrocytes have been described in which it may be possible to correlate the release of MVs with expression of alkaline phosphatase or other markers of differentiation by the cultured cells. In the case of rat chondrocytes, it was possible to quantify the yield of calcifiable MVs [21]. Thus, it may be possible to quantitatively relate the effect of chondrocyte differentiation on the rate of MV biogenesis.

In growth plates, it also has been suggested that MV biogenesis is the result of programmed cell death (apoptosis) [5••]. This hypothesis would not account for MV biogenesis occurring in newly formed bone and dentin, where most MV-releasing osteoblasts and odontoblasts appear viable. However, in the deepest layers of the growth plate, most chondrocytes exhibit a hydropic form of programmed cell death [22]. Furthermore, cytoplasmic blebbing and vesiculation is a well-known feature of the apoptotic process that can be triggered in chondrocytes by increased intracellular Ca^{2+} [23]. Recent studies from the author's laboratory have demonstrated positive TUNEL stains (for apoptotic DNA fragmentation) in the nuclei of approximately one third of proliferating zone chondrocytes, two thirds of the nuclei of early hypertrophic and maturing chondrocytes (the level where MVs arise), and in virtually all late hypertrophic chondrocytes in growth plates of 3-week-old, rapidly growing rats. These data suggest that growth plate chondrocytes release MVs during an early stage of apoptosis. Later, the deep hypertrophic chondrocytes undergo hydropic cellular disruption, that is, a morphologic pattern unlike that of classical apoptosis [22]. Although the majority of chondrocytes in the growth plate die and are reabsorbed during vascular invasion from the underlying metaphysis [6], there is accumulating evidence indicating that the occasional chondrocyte may bypass apoptosis, and through a process of asymmetric cell division persist as an osteoblast [24].

Mechanism of Matrix Vesicle Calcification

There is considerable evidence indicating that MVs initiate the first crystals of hydroxyapatite (HA) bone mineral [5••]. During phase I of mineral initiation, Ca^{2+} is attracted to MVs by calcium-binding molecules that are concentrated in the MV structure. These include the following: 1) calcium-binding acidic phospholipids, especially phosphatidyl serine (PS) [25], which is concentrated at the inner surface of the MV membrane, thus promoting the ingress of Ca^{2+}; and 2) calcium-binding proteins enriched in MVs, including annexin II (calpactin), annexin V (anchorin CII), and annexin VI plus calbindin D9K (*see* [5••]). Annexins also can function as transmembrane Ca^{2+} channels [5••]. The local intra- and perivesicular PO_4^{3-} concentration is raised by the enzymatic activity of phosphohydrolases that are enriched in the MV membrane, especially alkaline phosphatase [26], adenosine monophosphate phosphodiesterase [26], adenosine triphosphatase [27], and nucleoside triphosphate pyrophosphohydrolase [28]. Uptake of PO_4^{3-} also is facilitated by the action of a sodium-dependent PO_4-transporter that is present in MVs [29]. Elevation of Ca^{2+} and PO_4^{3-} within the protective microenvironment of the matrix vesicle sap, when exceeding the solubility product of calcium and PO_4 ions, may lead to precipitation of the first $CaPO_4$ mineral deposits near the inner surface of the MV membrane where Ca^{2+}- and PO_4^{3-}-concentrating molecules coincide. The intravesicular pH may be raised by the action of carbonic anhydrase that is concentrated in MVs [30], and could stabilize the initial mineral crystals.

Phase II, the propagation phase of MV mineralization, begins with the release of preformed HA crystals into the extravesicular fluid. In normal animals, the extracellular fluid bathing MVs contains homeostatically maintained levels of calcium and PO_4 sufficient to support continued nucleation of new HA crystals on preformed HA templates. Crystal perforation of the MV membranes would be augmented by phospholipases [31] and proteases [32] that are present within MVs. Propagation of mineral into the perivesicular matrix would be facilitated by metalloproteinases of MVs, which are capable of degrading mineral-inhibiting proteoglycans [33]. Electron microscopy of calcified areas of growth plate cartilage matrix shows that MVs are broken apart and degraded during advanced stages of mineral propagation [19].

During initial mineral propagation it is typical for HA crystals to form radial clusters at the periphery of MVs, not only in growth plate, but also in newly forming bone and predentin [5••]. Adjacent collagen fibrils play an important role in regulating the ensuing biomineralization cascade once this process has been initiated by MVs [34]. Recent work indicates that types II and X collagens are bound to the outer surfaces of MVs and may serve as a bridge for crystal propagation out into the extravesicular matrix [35]. Collagen calcification would follow vesicle calcification, with mineral deposition occurring in and on

collagen fibrils in a spatially oriented array [34]. The vectorial sequence of mineral propagation from MVs to adjacent collagen is especially well illustrated in electron microscopic studies of mineralizing turkey tendon [34].

Matrix Vesicle Involvement in Osteoarthritis and Crystal Deposition Arthritis

The pioneering work of Ali and Wisby [36] showed that the pathogenesis of osteoarthritis is associated with excessive and uneven calcification of the deep, tide mark zone of articular cartilage. Matrix vesicles, isolated from the articular cartilage of patients with osteoarthritis were shown to possess a markedly increased alkaline phosphatase activity, and were more prone to initiate in vitro calcification [36,37]. These observations support the hypothesis that an irregular, hypercalcified, and physically hardened subchondral tide mark in osteoarthritis joints creates abnormal and excessive mechanical stress, which leads to premature erosion of the overlying articular cartilage.

Recent studies of osteoarthritis cartilage also have demonstrated an abnormally increased rate of chondrocyte maturation [38] and apoptosis [39]. Premature maturation of chondrocytes in osteoarthritis would be expected to lead to the release of increased numbers of alkaline phosphatase–positive, mineralization-competent MVs. Furthermore, an excessive number of apoptotic chondrocytes in osteoarthritis would release more proteolytic enzymes into the matrix, especially matrix metalloproteinases [40] and cathepsins [41]. These enzymes digest proteoglycans, elastin, and collagen, thus provoking further degradation of the articular cartilage matrix, as well as further mineral propagation in the tide mark area because of the removal of mineral-inhibiting proteoglycans.

A unique and poorly understood feature of osteoarthritis is the overgrowth of subchondral bone and the formation of osteophyte bone spurs at the edges of affected joints. It recently has been shown by immunohistochemistry that articular chondrocytes express and synthesize BMPs [42]. Bone morphogenetic proteins are capable of inducing ectopic bone formation, and they also promote normal chondrocyte maturation [18•] and apoptosis [43]. Bone morphogenetic proteins generated by prematurely differentiated osteoarthritis chondrocytes, and released into the matrix in MVs [44], could stimulate the overgrowth of subchondral bone and the formation of periarticular osteophytes.

Calcium pyrophosphate deposition disease, also known as chondrocalcinosis and pseudogout, is the most prevalent form of crystal deposition arthritis. This disease results from the deposition of calcium pyrophosphate crystals into midzone articular cartilage. The enzyme that generates PPi, nucleoside triphosphate pyrophosphohydrolase (NTPPH), is concentrated in the membranes of chondrocytes and MVs [1,28]. Under normal conditions, the PPi that is generated by NTPPH is broken down to inorganic phosphate by the alkaline phosphatase of MVs and chondrocytes [45,46]. However, in calcium pyrophosphate deposition arthritis, it appears that the balance of PPi formation versus PPi hydrolysis is tilted toward excessive PPi formation [47], thus leading to CaPPi deposition.

Crystal deposition arthritis caused by hydroxyapatite deposition is a feature of calcifying tendonitis [48] and Milwaukee shoulder syndrome [49]. In calcifying tendonitis, tenocytes undergo chondrogenic differentiation and generate a cartilage-like matrix containing MVs [48]. These MVs then initiate HA deposition as in growth plate [19] and turkey tendon [34]. The possible role of an altered enzymatic balance favoring alkaline phosphatase over NTPPH has not been extensively investigated in human tendonitis. However, a potential new experimental model of chondroosseous metaplasia of tendons, associated with apatite deposition in joints, is presented in the phenotype of "tiptoe walking" mice. These mice have a naturally occurring null mutation of the gene for NTPPH [50], which leads to excessive HA deposits in articular cartilage and spinal ligaments. In "tiptoe walking" mice, it appears that excessive calcification of joints and tendons results from unopposed alkaline phosphatase–mediated generation of Pi caused by a deficiency of NTPPH, which normally generates sufficient mineral-inhibiting PPi to prevent excessive calcium Pi deposition. A similar extracellular PPi deficiency, associated with hypermineralization of articular cartilage and spinal ligaments, is seen in *ank*-deficient mice. The *ank* gene specifies the synthesis of an outwardly directed plasma membrane PPi transporter, which functions to maintain normal levels of extracellular PPi [51]. In *ank*-deficient mice the level of extracellular PPi falls, resulting in excessive, alkaline phosphatase-mediated HA deposition in articular cartilage and tendons [52]. In personal communication, Sampson [52] has indicated to the author that mineralization in *ank*/*ank* mice is initiated in association with MVs.

Conclusions

Matrix vesicles are released by chondrocytes, osteoblasts, odontoblasts, and tenocytes into selected sites where matrix calcification will occur. Matrix vesicles initiate deposition of the first crystals of apatitic bone mineral through the interaction of phosphohydrolase enzymes, especially alkaline phosphatase and NTPPH, plus calcium-binding molecules, all of which are enriched in MVs. Once initiated within MVs, apatitic mineral crystals replicate through a process of homologous nucleation and spread by propagation to involve large areas of the extravesicular matrix. The rate of mineral propagation is controlled by homeostatically supplied extracellular calcium and PO_4 levels plus the extravesicular concentration of naturally occurring inhibitors of mineral propagation, notably PPi.

In osteoarthritis, hydroxyapatite deposition arthritis (calcifying tendonitis) and ankylosing spondylitis of

humans and of "tiptoe walking" and *ank/ank* mice, MVs initiate pathologic articular calcification, which, in turn, augments disease progression. Advanced chondrocyte differentiation or apoptosis of chondrocytes in osteoarthritis articular cartilage promotes the release of greater numbers of alkaline phosphatase–enriched MVs, which, in turn, cause excessive and irregular calcification of the deep articular cartilage and excessive erosion of the overlying superficial cartilage. The release of BMPs by apoptotic articular chondrocytes into the matrix or encapsulated in MVs may promote the hyperostosis of subchondral bone and the formation of osteophytic bone spurs typical of osteoarthritis.

Acknowledgments

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