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Biomineralization and matrix vesicles in biology and pathology

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Abstract

In normal healthy individuals, mineral formation is restricted to specialized tissues which form the skeleton and the dentition. Within these tissues, mineral formation is tightly controlled both in growth and development and in normal adult life. The mechanism of calcification in skeletal and dental tissues has been under investigation for a considerable period. One feature common to almost all of these normal mineralization mechanisms is the elaboration of matrix vesicles, small (20–200 nm) membrane particles, which bud off from the plasma membrane of mineralizing cells and are released into the pre-mineralized organic matrix. The first crystals which form on this organic matrix are seen in and around matrix vesicles. Pathologic ectopic mineralization is seen in a number of human genetic and acquired diseases, including calcification of joint cartilage resulting in osteoarthritis and mineralization of the cardiovasculature resulting in exacerbation of atherosclerosis and blockage of blood vessels. Surprisingly, increasing evidence supports the contention that the mechanisms of soft tissue calcification are similar to those seen in normal skeletal development. In particular, matrix vesicle-like membranes are observed in a number of ectopic calcifications. The purpose of this review is to describe how matrix vesicles function in normal mineral formation and review the evidence for their participation in pathologic calcification.

Introduction

The mechanisms involved in the formation of mineralized tissues have been under investigation for many years. While much progress has been made in this period, much still remains to be discovered. One surprising outcome from this research is the notion that pathological calcification in normal soft tissues may utilize mechanisms similar to those found in hard tissues. A key constituent found in both normal and ectopic calcifications are matrix vesicles (MVs) and MV-like particles. These small (20–200 nm) particles are budded off from the plasma membrane of chondrocytes, osteoblasts, and odontoblasts prior to the onset of matrix mineralization. Similar structures have been observed in soft tissue calcification. The purpose of this review is to first describe the role of MV in hard tissue mineralization and then to relate that information to pathological calcification.

The discovery of matrix vesicles

Ultrastructural studies of pre-mineralized matrices provided the first evidence that the initial crystals were formed in and around MV [1,2]. As is common in emerging science, these initial observations were greeted with considerable skepticism. Some authorities dismissed them as preparation artifacts, which were rarely seen when the tissue was prepared anhydrously [3]. Over time, considerable evidence collected in vitro and in vivo

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substantiated the role of MV in calcification of bone, cartilage, and dentin [4-9]. Indeed, even some of the skeptics began to report the existence and function of MV [10]. By the mid 1980s, the study of MV in the mineralization of hard tissues blossomed. A plethora of studies were carried out to ascertain how MVs are formed and how they participated in the mineralization process. While these studies are by no means complete, a cohesive picture of MV action is emerging.

Composition of matrix vesicles

MV are budded from the plasma membranes of mineral-forming cells and oriented so that the exterior face of MV is the same as that of the parent membrane [11-13]. The composition of MV is, however, different from that of the membranes from which they originate. The principle components of MV are shown in Table 1. MVs are significantly enriched in tissue nonspecific alkaline phosphatase (TNAP) and phosphatidylserine compared to the cell plasma membrane [5,14-18]. Other important components include the annexins, nucleotide pyrophosphatase phosphodiesterase 1 (NPP1; PC1), the type III Na^+/PO_4^{3-} cotransporters Pit1,2 and Phospho-1, a phosphatase highly specific for phosphocholine and phosphoethanolamine [19-22]. The mechanism of MV biogenesis is not fully understood, but one component of the mechanism involves the formation of cholesterol-rich lipid rafts in the parent membrane prior to MV formation [23,24]. Each of the components in the table has been linked to key functions of MV and will be highlighted below.

The biochemistry and cell biology of mineralization

The vertebrate skeleton produces five different mineralized tissues: dental enamel, dentin, cementum, calcified cartilage, and bone. The composition of these tissues is shown in Table 2. While the mineral content in each of these tissues varies, the mineralized phase is the same, a carbonate-rich, hydroxyapatite, containing small amounts of Na^+, K^+,Mg^{+2}, and Cl^-, termed biological apatite [25,26]. The organic phase of these tissues is composed primarily of collagens, except in enamel. This most highly mineralized tissue found on the exterior surface of the teeth is produced by a unique mechanism, different from that of the other four tissues. Consideration of this mechanism is outside the scope of this review; further information can be found in these recent reviews [27,28].

Of the collagenous mineralized tissues, three are composed of mainly type I collagen, the exception being calcified cartilage which is built on a type II collagen matrix. All of the collagenous mineralized tissue matrices also contain non-collagenous proteins, whose function in mineralization and tissue function is a major research area in hard tissue biology. Some of the important non-collagenous proteins in mineralized tissues are listed in Table 2. The overall mechanism of mineralization of the four collagenous tissues is the same, cells secrete an unmineralized organic matrix and the mineral phase then forms in the space between the cells and the mineralization front (see Fig. 1). In the figure, OB labels an osteoblast, MF shows the mineralization front, C labels collagen fibrils, and MV points to a matrix vesicle with crystals forming in/around it. As can be seen in this image, there is a space between the osteoblasts and the mineralization front, which is composed of the organic matrix. In addition to synthesizing and secreting the organic matrix, the cells maintain the ion composition of the extracellular space through the action of Ca^{2+} and PO_4^{3-} ion pumps [29,30].

The activity of these ions in the extracellular space is modulated by non-collagenous matrix proteins and the presence of inorganic pyrophosphate (POP). The latter compound, at low concentrations, is a potent inhibitor of mineral formation. Non-collagenous proteins have been shown to enhance mineral formation, inhibit mineral formation, or do both [31-39]

Thus, in the pre-mineralized matrix, conditions are poised for mineral to form, but the process is blocked by the inhibitors. It is currently believed that mineralization is triggered by alteration of the PO_4^{3-}/POP ratio [40]. This formulation is schematized in Fig. 2.

The figure indicates the interactions of the cell (osteoblast, odonotoblast, or chondrocyte) and MV in regulating the extracellular concentrations of Ca^{2+} and PO_4^{3-}. As shown in the figure, cells pump Ca^{2+} out into the extracellular space. The cytoplasmic calcium concentration is approximately 0.1 μM. The origin of the matrix Ca^{2+} is from the blood, which is regulated to a fixed high level (2.5 mM total, ~1 mM ionic) by the calcium homeostasis system [41]. The ionic concentration of calcium in the extracellular space is modulated by the presence of non-collagenous matrix proteins [42,43]. In contrast, cellular PO_4^{3-} levels are in the range of 5 mM as PO_4^{3-} is required for many metabolic reactions. Keeping the cytoplasmic Ca^{2+} concentration low allows the cytoplasmic concentration of PO_4^{3-} to be high without causing mineralization to occur intracellularly. The intracellular phosphate pool is fed by PO_4^{3-} uptake from the blood and adenosine triphosphate (ATP) and AMP hydrolysis. POP is formed in a number of ATP requiring reactions, and some of these POPs are hydrolyzed to PO_4^{3-} by the enzyme pyrophosphatase. Some of the POPs, however, are transported into the matrix via the progressive ankylosis protein (ANK) [44-46]. The importance of this protein in regulating mineralization became evident when it was discovered that loss of function mutations in ANK resulted in hypermineralization [44-47]. MVs play a key role in the maintenance of the PO_4^{3-}/POP ratio in the matrix because they contain two key enzymes which regulate this ratio: NPP1 which hydrolyzes extracellular nucleoside triphosphates to increase extracellular POP concentration and TNAP, which decreases POP and increases PO_4^{3-} [44-48]. Upregulation of TNAP is a key developmental event in mineralization, and loss of function mutations in TNAP result in hypophosphatasia, characterized by undermineralization [49-54]. Systematic analysis of transgenic mice with deletions of NPP1, TNAP, and ANK has resulted in our current understanding of the regulatory role of the PO_4^{3-}/POP ratio in controlling the onset of mineral formation [40,55,56].

Role of collagens in mineralization

The endpoint for matrix mineralization is the deposition of small crystallites into collagen fibrils probably at the hole zones in the collagen structure [57,58]. It has been shown that the classical Hodge–Petruska quarter staggered array of collagen molecules can be arranged so that the hole zones are aligned to form channels large enough to accommodate nanocrystals [58,59]. In early mineralization, apatite platelets become oriented so that their c-axes are parallel to the fiber axis; ultimately, all of the intrafibrillar spaces are filled with mineral, resulting in a flexing of collagen molecules away from the fiber axis [60,61]. Additional observations using atomic force microscopy have provided evidence consistent with these models. For example, Tong, et al. have shown that the mineral in young bovine bone consists mainly of small apatite platelets (9×6×2 nm) which can be fit into aligned hole zones in the fibrillar structure as postulated by Katz and co-workers [57,62,63]. The conclusion from these studies is that small crystals can enter the fibril, probably via the hole zones and fibrillar pores, and that they further propagate within the fibril to fill all available spaces.

Mineral nucleation

The exact mechanism through which hydroxyapatite crystals form in vertebrate hard tissues has been widely debated [64-69]. The most likely mechanism proposed for bone and cartilage mineralization is based on the concept of heterogeneous nucleation [70]. This mechanism relies on organic or inorganic precursor seeds to direct the formation of apatite

from soluble inorganic ions. Substantial differences exist among authorities as to where this nucleation occurs and the exact molecular nature of the nucleator. One group of investigators propose that matrix vesicles are the site of initial or primary nucleation, as a prerequisite to subsequent secondary mineralization of the extracellular matrix [8,71-73]. In this scheme, Ca^{2+} and PO_4^{3-} in the extracellular matrix are transported into the interior of MV via MV transporters, annexins for Ca^{2+} and Pit-1,2 for PO_4^{3-} [74,75]. Within MV, the ions interact with phospholipids to form a nucleational core complex, resulting in the formation of nanocrystals [76-79]. How these nanocrystals exit the vesicle and reach the collagen fibrils is a matter of some debate. Propagation of the initial crystal seeds is thought to proceed by purely physical chemical means, based on the metastable nature of the extracellular fluid [38,80-82].

An alternative view questions the feasibility of this approach on physical chemical grounds, and proposes instead direct nucleation of apatite by matrix macromolecules, principally collagen, but possibly also involving phosphoproteins, phospholipids, and proteolipids [68,83-87]. Further, studies of the behavior of phosphoproteins in vitro are consistent with their role as nucleators or facilitators of nucleation [34,87]. While each of the major hypothesis for the initiation of mineralization is plausible and backed by a substantial body of evidence, neither hypothesis has been able to fully explain all of the known features of mineralized tissue calcification.

Summary of the role of MV in normal calcification

At present, MVs have two well-established roles in initiating matrix mineralization. The best and most accepted role for these membranes is their function in regulating the PO_4^{3-}/POP ratio in the pre-mineralized extracellular space. The data supporting this view have been discussed above, and while some questions remain as to the exact participation of each of the MV enzymes (NPP1, TNAP, and Phospho1) in the process, it seems clear that these enzymes are key players in the mineralization process. The second putative role for MV is to nucleate mineral crystals and thus begin the mineralization cascade which results in massive mineralization of the matrix. Evidence has been reviewed showing that MV lipids, and particularly, an MV complex, proposed by Wuthier and colleagues as well as Boskey and collaborators and Boyan and co-workers, are the nucleation sites in bone and calcifying cartilage [68,83,88-92]. As the initial crystals observed in mineralizing tissues have frequently been seen in and around MV, the role of membranes and membrane constituents in mineral formation cannot be ignored and will be seen to be a consistent feature of pathological calcifications.

Pathological ectopic calcification

The inappropriate formation of biological mineral in soft tissues is called ectopic calcification and is considered pathologic. In some instances of ectopic calcification, the mineral formed is hydroxyapatite, although other calcium salts (e.g., oxalate or octacalcium phosphate) are formed in kidney stones, for example. Calcification in joints results in osteoarthritis, and mineralization in the cardiovascular system (as well as in cardiovascular prostheses) is especially troubling, resulting in increased morbidity and mortality [93]. Almost as soon as the existence of MV in mineralizing tissues was determined, and their putative role in normal calcification proposed, reports of MV and MV-like particles in abnormal calcification began to appear [94]. Subsequent studies of vascular calcification have resulted in the surprising finding that the molecular events associated with calcification of the vasculature resemble endochondral ossification of bone [93,95]. The underlying mechanisms resulting in cardiovascular calcification derive from observations that vessel cells, including periocytes, vascular endothelial cells, and myofibroblasts can undergo

aspects of osteoblastic differentiation. In particular, upregulation of TNAP and the production of MV-like particles have been observed [96,97]. While the progression of vascular calcification appears to follow the well-known biomineralization pathway, the signals which initiate this process are less well understood. Genetic diseases which result in ectopic mineralization have been shown to derive from either the absence of a circulating mineralization inhibitor, such as matrix gla protein, or from altered signaling, as is the case in fibrodysplasia ossificans progressiva [98-102]. Why mineralization begins in the absence of such mutations is less clear. A number of factors which have been proposed for the stimulation of vascular calcification include oxidative stress, hyperlipidemia, and inflammation [93,103]. Whatever initiates the process, MV or MV-like particles appear to play a role in vascular calcification, as they do in normal hard tissue biogenesis.

Evidence for the occurrence of MV in ectopic calcification

As indicated above, MV-like particles were seen by ultrastructural analyses in arthritic cartilage [94,104,105]. Further studies in vitro have substantiated the hypothesis that these membraneous bodies are very similar or identical to MV [105-107]. It is also well-known that chondrocyte apoptosis is seen in arthritic cartilage and that chondrocyte apoptosis is a prominent feature of calcifying cartilage [108,109]. One question which might be asked is whether the membraneous structures seen in ectopic calcification arise either from necrotic membrane fragmentation or are the results of chondrocyte apoptosis. To answer this question, we cultured chondrocytes from embryonic chick tibia and measured the release of MV from the cells. We also measured the release of lactate dehydrogenase as a measure of cell fragmentation [110]. Under these conditions, MV release was accompanied by minimal release of cell contents. In contrast, cytotoxic agents resulted in massive cell fragmentation. The membranes released in this process could be distinguished from authentic MV by the presence of marker enzymes not associated with MV. Subsequently, Kirsch has clearly shown that in growth plate chondrocytes, MVs are not apoptotic bodies [111]. Although similar studies have not yet been done with cells from soft tissue calcification sites, MV-like particles have also been observed in vascular calcification [97,112,113]. In addition, cultured blood vessel smooth muscle cells (BVSMC) have been demonstrated to produce vesicles in culture, which are very similar in content to those produced by chondrocytes [97,114]. The similarity of BVSMC vesicle composition and vesicles seen in arthritic and arthritic calcification to the composition of skeletal MV suggests that they act via similar mechanisms to those seen in hard tissues: they modulate the local PO_4^{3-}/POP ratio and they provide nucleation sites for the initial crystals. For example, Millan and co-workers have shown that TNAP inhibitors block calcification of vascular smooth muscle cells, which suggests parallels to the role of this enzyme in skeletal mineralization [115].

What can be learned about ectopic calcification from consideration of normal mineralization mechanisms?

Most structures in the vertebrate body are in ionic equilibrium with the blood, and the blood is a metastable solution of Ca^{2+} and PO_4^{3-} [82]. Mineral doesn't form all over the body because there are mineralization inhibitors in the blood and elsewhere which stabilize the metastable solution. In normal skeletal development, MV functions to remove one potent inhibitor of calcification, POP. Therefore, once cells begin to produce MVs, they can promote mineral formation. What causes articular chondrocytes or vascular cells to begin this process? Pathogenic signals in the form of inflammation, hyperlipidemia, and stress are the likely culprits, and it appears that they act by stimulating a signaling cascade not unlike that seen in the skeleton. Local release of pro-calcification molecules like BMPs reprogram cells of mesenchymal origin to begin recapitulating endochondral ossification. One consequence of this mechanism is the elaboration of MV. Thus, by better understanding the

role of MVs in normal mineralization, we can better deal with them in pathologic calcification.

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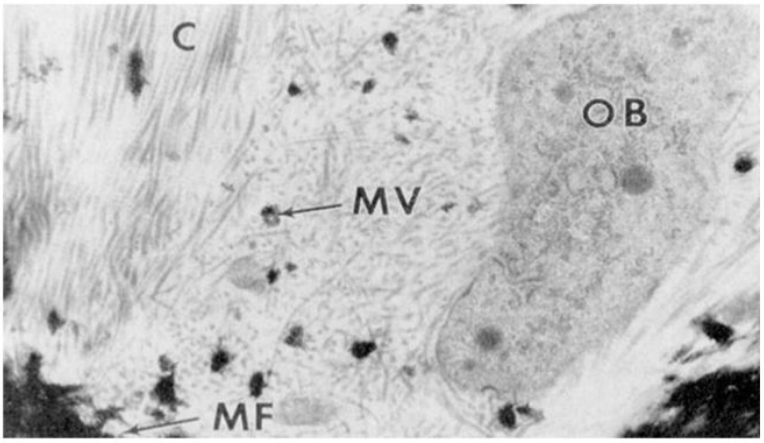

Fig. 1.

Transmission electron micrograph of the mineralization front (*MF*) in forming bone. An osteoblast (*OB*) is secreting matrix including collagen (*C*). A matrix vesicle (*MV*) with mineral is seen in the matrix

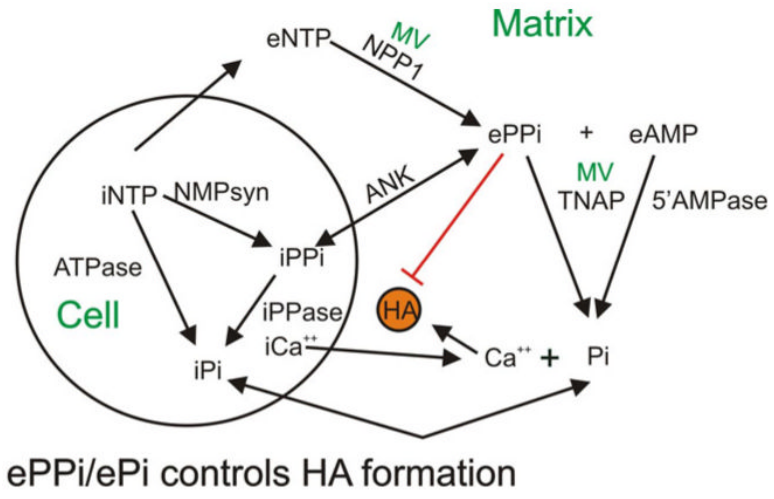

Fig. 2.

A schematic representation of the regulation of Ca^{2+} and Pi concentration in the extracellular matrix by cells and MV. Intracellular nucleotide triphosphates (*iNTP*) contribute to the intracellular Pi pool through enzymatic hydrolysis (*ATPase*) and the intracellular pyrophosphate pool (*iPPi*) by enzymes which produce nucleotide monophosphates (*NMPsyn*). iPPi also feeds the Pi pool by hydrolysis by intracellular pyrophosphatase (*iPPase*). iPPi is transported into the matrix via the progressive ankylosis protein (*ANK*). iNTPs also are secreted into the matrix to form a pool of extracellular NTPs (*eNTP*). eNTPs are converted by MV enzyme nucleotide pyrophosphohydrolase (*NPP1*) to ePPi, and ePPI is broken down to Pi by MV enzyme tissue nonspecific alkaline phosphatase (*TNAP*). In the matrix, Ca^{2+} and PI combine to form hydroxyapatite (*HA*)

Table 1

Major components of matrix vesicles

Enzymes
Alkaline phosphatase (TNAP)
Phospho-1
Na+/K+ATPase
NPP1/PC-1
MMP-2
MMP-3
MMP-13
Transport proteins
Annexins 5, 2, 6, 11, 4, 1, 7
Pit 1,2
Other proteins
Integrins β1, β5, αV, α11, α1, α3
Lipids
Neutrals
Free fatty acids
Phosphatidylcholine
Phosphatidylethanolamine
Phosphatidylinositol
Phosphatidylserine

Table 2

Mineralized tissue composition

Tissue	Mineral (%)	Collagen	Major non-collagen proteins
Enamel	95–98%	None	Amelogein Enamelin
Dentin	80–52%	Type I	Dentin phosphoprotein Dentin sialoprotein Dentin matrix protein 1
Cementum	50–70%	Type I	Sibling proteins[a] Osteocalcin
Bone	50–90%	Type I, III	Sibling proteins[a] Osteocalcin
Calcified cartilage	35–50%	Type II, IX, X	Proteoglycan (aggrecan)

[a]Sibling proteins are small integrin-binding ligand, N-linked glycoproteins and include osteopontin, bone sialoprotein, dentin matrix protein 1, dentinsialophosphoprotein, enamelin, and matrix extracellular phosphoglycoprotein