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Role of Matrix Vesicles in Biomineralization

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Summary

Matrix vesicles have been implicated in the mineralization of calcified cartilage, bone and dentin for more than 40 years. During this period, their exact role, if any in the nucleation of hydroxyapatite mineral, and its subsequent association with the collagen fibrils in the organic matrix has been debated and remains controversial. Several hypotheses have been recently introduced to explain in greater detail how matrix vesicles function in biomineralization. This review will summarize recent advances, and open questions in the ongoing saga of these interesting and enigmatic extracellular organelles.

Keywords

Matrix Vesicles; Calcification; Biomineralization; Alkaline Phosphatase; Hydroxyapatite; Nucleation

1. Introduction

The term matrix vesicles (MV) refers to small (20-200 nm) spherical bodies observed in the pre-mineralized matrix of dentin, cartilage and bone. They appear to be bounded by a lipid bilayer, and are often found associated with small crystals of calcium phosphate mineral. The purpose of this review is to summarize the properties of MV and to describe how they function in the process of mineralization. It should be emphasized that both the properties and the mechanism(s) through which MV function are active areas of investigation and in some contention. This summary is therefore a snapshot of current knowledge from one vantage point.

2. Matrix Vesicle Properties

MV were originally discovered through ultrastructural analysis of growth plate cartilage and in bone, where they were seen as the initial sites of mineral formation, prior to matrix mineralization [1-3]. Subsequent analysis revealed that they are derived from the plasma membrane of mineral forming cells (*i.e.* chondrocytes, osteoblasts, odontoblasts), but have a membrane composition different from the parent membrane [4-13]. In particular, MV are enriched in tissue non-specific alkaline phosphatase (TNAP), nucleotide pyrophosphatase phosphodiesterase (NPP1/PC-1), annexins (ANX; principally annexins II, V & VI) and phosphatidyl serine (PS) relative to the membranes from which they are derived (Table 1) [5, 12,14-21]. MV also contain matrix metalloproteinases (MMPs) [22-24]. The acidic

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phospholipids can bind Ca^{2+} , but more importantly, we have shown that they facilitate calcium dependent annexin binding, and are permissive for annexins to form calcium channels through the membrane [14,25,26]. Recently, proteomic analysis of MV isolated from cartilage and osteoblast cultures have confirmed and extended the list of MV protein constituents to include proteoglycan link proteins and actin, a variety of integrins and PHOSPHO-1, a recently discovered PE/PC phosphatase known to be expressed in hypertrophic cartilage and mineralizing osteoblasts and in MV (Boesze-Battaglia, Harrison and Golub, unpublished observations)[27-30].

TNAP is the best studied of the MV proteins, and has been implicated in mineralization mechanisms since the 1920's [31]. A plethora of studies have confirmed the importance of TNAP in initiating mineral formation [32-34]. Mutations in TNAP which decrease its activity result in the genetic disease, hypophosphatasia, leading to undermineralization of bone; complete loss of TNAP activity is perinatal lethal. More recently, the role of NPP1 and pyrophosphate metabolism has come to the fore [35,36]. The occurrence of TNAP on the external surface of MV thus further emphasizes the centrality of these membranous particles in the calcification process [17]. Nonetheless, experiments carried out in our laboratories have shown that TNAP activity alone is not sufficient for normal mineralization, unless the enzyme is membrane anchored [37]. Thus, while regulation of extracellular PPi and Pi concentrations is clearly necessary for mineralization, TNAP appears to play additional roles in the process. By most accounts, MV constituents collaborate to initiate intravesicular mineral formation [38-42]. Some experts believe that these nanocrystals are released into the extravesicular space, where they are postulated to both seed additional crystals, and to mineralize collagen fibrils [39,40,42].

Work from our laboratories and others has demonstrated the importance of the membrane proteins known as annexins in MV function [14,26,39,43]. Annexins are Ca^{2+} and phospholipid binding proteins which, under the right conditions can form calcium channels through MV membranes [26]. These conditions include membranes with a high content of acidic phospholipids. We have demonstrated that MV contain annexins II, V and VI, and in such membranes, MV annexins can form a hexameric structure which appears to be associated with Ca^{2+} transport. Moreover, we have demonstrated that annexin V binds to Types II and X collagen [14]. MV also bind to collagens [14,44-46]. The best studied of these interactions is the binding of MV to the N-telopeptide of Type II collagen via MV associated annexin V [44,47]. In earlier work, Wu and coworkers provided evidence for MV binding to native, but not denatured Type I collagen via interaction with several MV proteins [46].

3. Role of MV Constituents in Mineralization

That MV play some role in the calcification of cartilage, bone and dentin is now generally accepted, although the exact nature and extent of that role remains controversial, and the mechanisms through which MV initiate matrix mineralization in these tissues remains unclear. An emerging consensus now emphasizes the central role of the MV enzymes TNAP and NPP1, in conjunction with the cell associated ankylosis protein (ANK) in regulating the onset of calcification [17,36,48,49]. The currently held doctrine posits that the activities of ANK and NPP1 function to suppress mineralization by increasing the extracellular concentration of the calcification inhibitor, pyrophosphate (PPi), while TNAP functions to promote mineralization by decreasing the concentration of PPi and increasing the concentration of the mineralization promoter, inorganic phosphate (Pi) (Table 2) [35,50-53]. In this scheme, extracellular PPi is formed from extracellular nucleoside triphosphates (NTP) by NPP1 and exported from cells through the action of ANK; it is hydrolyzed to Pi by TNAP.

Early studies of MV lipid composition determined that there were significant differences between the lipid content of MV and the plasma membranes from which they arise. Wuthier and colleagues cataloged these differences, and hypothesized that the increase in acidic phospholipids in MV was somehow associated with MV calcification [12,54,55]. Further studies from the Wuthier laboratory, along with those of Boyan and colleagues proposed that MV lipids could act as a nucleation site for hydroxyapatite formation [20,56,57]. This concept has been extensively developed under the concept of “nucleational core complex”, which describes the interactions of MV phospholipids, Ca^{2+} , PO_4^{3-} and some MV proteins to form a molecular architecture which nucleates apatite crystallization [58-62].

4. Matrix Vesicle Biogenesis

Morphological evidence supports the view that MV arise from cells by a “budding” process. In contrast, one study carried out in hybrid osteosarcoma cells concluded, “that matrix vesicle biogenesis is independent and distinct from that of plasma membrane biogenesis.” [63]. This conclusion has not yet been generally accepted. Alternatively, the notion that MV arise from the plasma membrane is supported by comparative lipid and protein studies of the vesicle and the plasma membrane of the epiphyseal chondrocyte and osteoblasts. From these investigations, subtle structural differences in the composition of the two membranes suggest that MV originate from specific sites on the hypertrophic chondrocyte plasma membrane (PM). It has been shown that MV contain higher concentrations of acidic phospholipids (phosphatidylserine, PS and phosphatidylinositol, PI) than the chondrocyte PM [15]. Overall, chondrocyte membranes contain similar components to matrix vesicles, but in different proportions.

Recently we studied the lipid organization of MV and PM [64]. In this study, we determined that chick growth plate MV were highly enriched in membrane raft microdomains containing high levels of cholesterol, glycosphosphatidylinositol GPI-anchored TNAP and phosphatidylserine (PS) localized to the external leaflet of the bilayer. To determine how such membrane microdomains arise during chondrocyte maturation we explored the role of PM cholesterol-dependent lipid-assemblies, in regulating the activities of lipid translocators involved in the externalization of PS. We first isolated and determined the composition of detergent resistant membranes (DRMs) from chondrocyte PM. DRMs isolated from chondrocyte PM are enriched in Ganglioside I (GM1) and cholesterol as well as GPI-anchored TNAP. Furthermore, these membrane domains are enriched in PS (localized to the external leaflet of the bilayer) and had significantly higher TNAP activity than non-cholesterol enriched domains. To understand the role of cholesterol-dependent lipid assemblies in the externalization of PS we measured the activities of two lipid transporters involved in PS externalization, aminophospholipid translocase (APLT) and phospholipid scramblase (PLSCR1) during maturation of a murine chondrocytic cell line, N1511. In this study, we provided the first evidence that maturing chondrocytes express PLSCR1 and have scramblase activity.

In bone, MV are also thought to arise by budding from the osteoblast plasma membrane [39]. Fedde, while studying MV released by the human osteosarcoma cell line, Saos-2, concluded, “MVs originate from specialized regions of the PM and are released in the same orientation as the PM” [65]. In SaOSLM2 osteoblastic cells, Gillette and Nielsen-Preiss found that annexin 2 and TNAP were co-localized to lipid raft microdomains [66]. A very recent study using this cell line concluded that MV arise from microvilli seen at the apical plasma membrane [67]. This finding is reminiscent of much earlier studies reported by Ornoy and Hale [68,69]. At this time it is not clear how the specialized membrane domains observed in chondrocytes and the microvilli budding process are related.

5. Matrix Mineralization

The organic phase of bone, dentin, cementum and calcified cartilage is dominated by collagen fibrils, which serve as the scaffold for calcification. In addition to collagen, the pre-mineralized matrix contains proteoglycans and acidic non-collagenous proteins which are believed to assist and modulate collagen calcification. The principal non-collagenous proteins of bone can be classified as glycosaminoglycan-containing molecules, the proteoglycans, glycoproteins, and α -carboxyglutamic acid-containing molecules, and phosphoproteins [42]. Of these, recent interest has focused on a subset of these proteins termed SIBLING's, an acronym for Small Integrin-Binding LIgand, N-linked Glycoprotein. The SIBLING's include osteopontin (OPN), bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP), enamelin and matrix extracellular phosphoglycoprotein (MEPE) [70]. Some of these proteins have been studied for their role in promoting or inhibiting mineralization *in vivo* and *in vitro*. DMP1, BSP and DSPP appear to promote mineralization, while OPN has been reported to exhibit both behaviors [71-79].

In calcifying cartilage, Type X collagen, fibronectin, matrix gla protein and transglutaminase 2 have been implicated in modulating mineralization [80-86]. Many studies have attempted to elucidate the role of matrix components in the mineralization process, and the structural basis for that role. Some generalizations have emerged: 1) many of the extracellular matrix protein are highly acidic, fueling speculation that they bind Ca^{2+} , and are therefore postulated to either inhibit calcification or serve as nucleators; 2) many matrix proteins are highly phosphorylated, contributing to their acidity, providing phosphate-rich nucleation sites, and serving as putative substrates for matrix phosphatases (*e.g.* TNAP) and 3) some matrix proteins have well defined cell binding domains (*e.g.* RGD) [70,73,86-89]. The exact mechanisms through which these proteins modulate mineral formation is not yet clear, but there is little doubt that they play key roles in the process.

The endpoint for matrix mineralization is the deposition of small crystallites into collagen fibrils, probably at the hole zones in the collagen structure [90,91]. 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 (see Figure 1) [92,93]. In early mineralization, apatite platelets become oriented so that their c-axes are parallel to the fiber axis; ultimately all of the intrafibrillar space is filled with mineral, resulting in a flexing of collagen molecules away from the fiber axis [90,94,95]. More recent observations using atomic force microscopy (AFM) 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 ($9 \times 6 \times 2$ nm), which can be fit into aligned hole zones in the fibrillar structure as postulated by Katz and co-workers [93]. 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 space. The exact mechanism through which hydroxyapatite crystals form in vertebrate hard tissues has been widely debated [39,96-101]. The most likely mechanism proposed for bone and cartilage mineralization is based on the concept of heterogeneous nucleation [102]. 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 [39,103-105]. 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 [99,106-110]. Further, studies of the behavior of phosphoproteins *in vitro* is consistent with their role as nucleators or facilitators of nucleation

[111,112]. Additional proteins thought to play key roles in the mineralization process include BAG-75, fetuin and DMP-1 [113-115]. While each of the major hypotheses for the initiation of mineralization is plausible and backed by a substantial body of evidence, none has been able to fully explain all of the known features of cartilage and bone calcification.

6. Role of MV in Mineral Nucleation

It is currently believed that MV have at least two principal roles in initiating calcification: 1) MV enzymes regulate the ratio of Pi to PPi in the extracellular fluid, and 2) MV proteins and lipids, including acidic phospholipids serve as nucleation sites for apatite deposition [39,42]. PPi, derived both from NPP1 catalyzed hydrolysis of extracellular NTP and from intracellular PPi transported via ANK, inhibits matrix mineralization, but not intravesicular mineral formation [116]. This inhibition is released through the action of TNAP, which hydrolyzes PPi, thus simultaneously removing the inhibitor and providing additional Pi for mineral formation (see Table 1) [35].

Thus, mineralization is said to proceed in two phases: an initial formation of apatite within MV, and a subsequent propagation phase in the matrix [35]. In this formulation, Ca^{2+} enters MV via an annexin channel and phosphate enters via a type III Na^+ dependent phosphate transporter to form apatite within MV [117-119]. Acidic phospholipids and other MV components are thought to nucleate these intravesicular nanocrystals [25,39,42,120-122]. Subsequently, the intravesicular mineral seeds matrix macromolecules, principally collagens.

These proposed mechanisms are presented schematically in Figure 2. In panel A, The MV are depicted as solely involved in the regulation of matrix ion concentrations. The inhibitor PPi is hydrolyzed by TNAP, to form Pi. The Ca^{2+} and PO_4^{3-} in the matrix can then initiate mineral formation at macromolecular nucleation sites as described above. In panel B, MV TNAP hydrolyzes PPi to Pi. Ca^{2+} and PO_4^{3-} ions enter MV and initiate mineral formation inside MV. As depicted in Figure 2 panel B, the crystals formed inside MV migrate to collagen, become inserted into the aligned hole zones of the fibrils, and then undergo maturation in the presence of extracellular Ca^{2+} and PO_4^{3-} ions and the reduced PPi concentration. In panel C of Figure 2, all of the events depicted in panel B also are postulated to occur, but in addition, the physical interaction of MV with collagen fibrils facilitates the deposition of mineral into the fibrils. In this formulation, specific binding of MV to fibrils allows insertion of crystal into the fibrillar structure. In addition, MV associated MMPs may provide localized remodeling of the fibrils to expedite crystal entry (see below). Maturation is then expected to proceed as previously described. The latter hypothesis, depicted in panel C is being actively investigated in my laboratory.

7. Matrix Metalloproteinases - Tissue Remodeling and Mineralization

Many reports have implicated MMPs in pathological mineralization associated with arthritis and cardiovascular calcification, but MMPs also appear to be important for normal tissue development and remodeling [123-131]. Moreover, MMPs have been localized to MV, where they may play significant roles in growth plate maturation and development [23]. In particular, matrix metalloproteinases are expressed in growth plate cartilage, are associated with MV, and are required for the developmental processes which result in calcification of the growth plate, and its subsequent replacement by endochondral bone [24,132-135]. These data are consistent with recent work by Inada, *et al.* who reported on the effects of a null mutation in MMP-13 (collagenase-3) and concluded that this mutation resulted in abnormalities in growth plate development, including delayed ossification [125].

Tissue remodeling is a well known feature in development, which is closely associated with the expression of matrix degrading enzymes [136-140]. Tissue invasiveness by tumors also

requires collagenolysis, and in cancer cells, enzymes which degrade collagen are often found associated with vesicles [141-143]. Mineralizing tissues also express matrix degrading enzymes which appear to be essential for the calcification process [144-146]. In addition to degrading collagen, gelatinases associated with MV can also degrade proteoglycans, which has been postulated to facilitate mineralization [147-150].

A number of MMPs are expressed in growth plate cartilage, including MMP-1, 2, 3 and 9, and the expression of these enzymes is regulated by 1,25-(OH)₂-vitamin D, retinoic acid, TGF α , and through integrin signaling [150-154]. Of particular interest is MMP-13, which is expressed in bone and cartilage, and appears to be under control of the transcriptional regulator, Cbfa1, the master regulator of bone formation [155]. Further, evidence is now accumulating that MMP's regulate growth factor economy in cartilage. For example, Boyan and Schwartz have recently shown that activation of MV MMP's by 1,25(OH)₂D₃ results in release of active growth factors from the cartilage matrix [156].

It has been known for many years that triple-helical collagen molecules are resistant to proteolysis by ordinary proteases, and instead that specific collagenases are required to degrade the molecules. Vertebrate collagenases specifically split Type I collagen between Gly⁷⁷⁵ and Ile⁷⁷⁶ in the α 1(I) chain and Gly⁷⁷⁵ and Leu⁷⁷⁶ in the α 2(I) chain, resulting in a larger fragment and a smaller fragment, the larger piece consisting of approximately 75% of the molecule and including the N-terminus [157,158]. MMP-13 can also release the N-terminal telopeptide of Type I collagen [158]. Collagenases cleave Type II collagen in a similar way between Gly⁹⁷⁵ & Leu⁹⁷⁶, in each of the 3 α -chains, although additional cleavages may also occur [159]. MMP-13 cleaves Type II collagen and also gelatin 40-fold more effectively than MMP-1. Once the initial cleavage by the collagenases is accomplished, the triple helical molecules denature at body temperature, and are susceptible to degradation by gelatinases, such as MMP-2 and 9, as well as other proteases.

8. Conclusions

Consideration of the data and hypotheses above leads to some distinct conclusions, and also leaves some open questions about the detailed role of MV in the mineralization of bone, cartilage and dentin. That they have some role(s) is now generally accepted. It is now understood that MV bud from the plasma membrane of mineral forming cells, but that they take with them a subset of the materials found in the parent membrane, leading to enrichment in the MV membrane of acidic phospholipids and certain proteins, particularly TNAP, NPP1 and Annexin V. In the case of TNAP, we estimate that the specific activity in cartilage MV is 10 $\times$ higher than in the PM from chondrocytes (Harrison and Golub, unpublished). It is also clear that the three proteins, TNAP, NPP1 and Annexin V have important roles in the process, although one report suggests that annexin V is not required for skeletal development [160]. As MV also contain other annexins, it is possible that the function of annexin V is shared among several of them [161]. It is also clear that MV participate in regulating the concentration of PPI in the matrix. Removal of PPI promotes mineral formation. There are also big questions about the mechanism which have yet to be answered: 1) if the MV nucleational core complex is the site of apatite formation, how do the apatite crystal find their way to the collagen fibrils? 2) How does annexin binding to collagen modulate the mineralization process? 3) What role, if any, do MV MMP's play in the mechanism? 4) What is the function of the non-collagenous matrix proteins? These and many other details of the mechanism remain to be elucidated.

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Figure 1. Cross sectional model of early collagen mineralization. The open circles represent triple helical collagen molecules and the large hatched circles represent mineral within the hole zone channels.

Figure 2.

Three possible mechanisms for the initiation of MV mediated matrix mineralization. In panel A, MV only regulate ion concentrations, leading to the formation of soluble molecular species which can initiate mineral formation in collagen fibrils. The circles represent MV, and the black lines depict mineral crystals. The double blue lines represent triple helical collagen molecules which are 300 nm in length. The dotted red line represents a dialysis membrane, which would not block mineralization by this mechanism. In panel B, MV regulate ion compositions leading to the formation of intravesicular apatite crystals, which leave the vesicle migrate to the mineralization front, where they seed collagen calcification. In panel C, MV associate directly with collagen, so that MV and matrix macromolecules cooperate to initiate matrix calcification.

Table 1

Principle Components of Matrix Vesicles

Proteins ^a	Lipids ^b	
<hr/>		
Enzymes:		MV/PM^c
Alkaline Phosphatase (TNAP)	Neutrals	0.63
Phospho-1	Free Fatty Acids	0.72
Na ⁺ /K ⁺ ATPase	Phosphatidylcholine	1.80
NPP1/PC-1	Phosphatidylethanolamine	1.90
MMP-2	Phosphatidylinositol	1.20
MMP-3	Phosphatidylserine	4.80
<hr/>		
MMP-13		
Transport proteins:		
Annexins 5, 2, 6, 11, 4, 1, 7		
Pit 1, 2		
Other Proteins:		
Integrins α_1 , α_5 , α_V , α_{11} , α_1 , α_3		

^aProtein composition based on the proteomic analyses [27,161-164], Boesze-Battaglia and Golub, unpublished, and the work of Boyan and Schwartz [156].

^bLipid composition based on the work of Wuthier and colleagues [12,55]

^cRelative abundance of lipids in MV as compared to plasma membrane.

Table 2

Matrix Pyrophosphate Effectors

Protein	Effector	Effect	Mineralization
NPP1, ANK	PPi	t	Inhibits
TNAP	PPi	9 Pi	tPromotes