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EXTRACELLULAR MATRIX VESICLES IN ENDOCHONDRAL BONE DEVELOPMENT AND IN HEALING AFTER INJURY

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Abstract

Matrix vesicles are fundamental components of initial mineral formation in endochondral and primary ossification. Regulation of their structure and function is multi-faceted and involves genomic and nongenomic pathways. Their function in mineralization may include matrix maturation and conveying cellular signals to the extracellular matrix as well. Intravesicular hydroxyapatite crystal formation is associated with primary mineralization; subsequent crystal formation, particularly at later stages, appears to be mediated by other mechanisms.

Key Words: matrix vesicles, calcification, mineralization, regulation, vitamin D, phospholipids, proteolipids, morphometry, chondrocyte, fluidity.

Introduction

Primary mineralization is regulated by chondrocytes, osteoblasts, and odontoblasts and is observed in epiphyseal cartilage, embryonic bone, post-fetal ossification, and repair and development of predentine, and bone induction. In all these instances a tight regulation of the process in both time and space is required.

Intravesicular crystals are seen prior to bulk phase mineral deposition, suggesting that matrix vesicles have a role in the initiation of calcification [1, 2, 8, 17, 59, 72]. While mineral crystals are first seen in matrix vesicles [3, 8, 14, 15, 72] it is unlikely that bulk phase mineral deposition requires vesicles or their constituents [35]. However, the involvement of matrix vesicles is believed to be an important factor in maintaining calcification cellular control. How this is accomplished will be discussed below.

The accepted method for isolation of matrix vesicles is by collagenase digestion of the calcifying tissue, followed by differential centrifugation [1, 4, 72, 74]. However, for frozen samples or tissues which are heterogeneous, this is not a useful approach. For these latter instances, tissue homogenization followed by density gradient centrifugation was found to be preferable [12, 30]. Using both differential centrifugation and/or density gradient centrifugation techniques, a number of biochemical characteristics of matrix vesicles have been identified. Matrix vesicles have lipid compositions distinct from the cell in general [47, 71] and from the plasma membrane [38, 60, 74]. Alkaline phosphatase, long associated with calcification [69] is the organelle’s marker enzyme [2, 9, 43]. Matrix vesicles continue to mature concomitant with maturation of the growth plate; for instance, their mineral ion composition changes [74] and proteolipid [22] and Ca-phospholipid-phosphate complex (CPLX) content [73] increases. Their composition and enzyme activity was shown to be regulated by hormones known to regulate calcification [25].

Although isolated matrix vesicles support hydroxyapatite formation in vitro [9, 72], a critical question in the biogenesis of matrix vesicles has been whether or not
they are "preloaded" with calcium and phosphate at the time of their release. Specific interactions of Ca\(^{2+}\) and phosphate ions with phospholipids in the cell membrane could result in the formation of CPLX [28]. Phospholipid-mineral interactions may be responsible for membrane fusion, allowing vesicular budding into the extracellular matrix [37, 72]. The occurrence of actin in isolated vesicle fractions is indicative of a cytoskeletal role in vesicle formation as well [45, 61, 72].

Whether multiple subclasses of matrix vesicles are produced by a single cell at a specific stage of differentiation or whether each cell produces only one class of vesicle is unknown. The continuing maturation of the vesicles in the extracellular matrix could also add to their apparent heterogeneity. Immediately after release, vesicles appear electron lucent, although Ca and phosphate may already be present. Following further uptake of calcium and phosphate, vesicle morphology is amorphous, homogeneous, and electron-opaque. Calcification on the internal surface of the membrane follows. Rupture of the vesicular membrane follows crystal growth. In the matrix, released crystals adhere to each other and serve as foci for continued crystal deposition, and calcospherite formation [59].

**Changes in Matrix Vesicle Morphology and Biochemistry in Bone Healing**

Removal of the marrow tissue in rat tibial bone has been developed as an animal model of primary mineralization during bone healing. Following injury, endosteal bone formation is activated. This process has been monitored as a function of time in perfusion-fixed tissue using computerized cytomorphometry [59, 60]. Matrix vesicles were sorted according to their type, diameter and distance from the calcified front [5, 6, 54, 59, 60, 62, 63]. Electron lucent vesicles were categorized as "empty"; those with amorphous electron opaque contents as "amorphous"; those containing crystalline structures as "crystalline"; and those with ruptured membranes and released crystals as "rupture" (Fig. 1).

These studies supported the hypothesis that cells are responsible for the production of electron lucent vesicles that continue to accumulate Ca and inorganic phosphorous (Pi), and acquire "amorphous-type" characteristics. Hydroxyapatite crystals form, followed by membrane rupture and exposure of the crystals to the matrix. Most vesicles were found within 3 µm from the calcified front. "Rupture" vesicles were closest to the front and had the largest diameter [59]. Matrix vesicle maturation on each day of healing was characterized by increased vesicular diameter and was accompanied by increased numbers of mature vesicles of the "crystal" and "rupture" types and decreased numbers of the "empty" and "amorphous" types [59].

Changes in matrix vesicle morphology correlate with biochemical changes in matrix vesicle-enriched microsomal fractions (MVEM) isolated from the healing tissue. At six days post-injury, there is an increase in MVEM alkaline phosphatase and phospholipase A2 specific activities (Fig. 2) [57]. Phospholipid composition changes with time. The amount of phosphatidylcholine decreases as a function of time. Phosphatidylserine content is greatest at days 6 and 14, which correlates with...
Matrix Vesicles

![Graph](image)

Figure 2: Specific activities of alkaline phosphatase expressed as µmol Pi/mg protein/minute and phospholipase A₂ expressed as percent hydrolysis/mg protein/minute in matrix vesicle-enriched membranes isolated from tibial bone healing following marrow ablation. Isolation is performed by differential centrifugation of homogenates of the newly formed tissues. Values are presented as means ± standard error of mean (S.E.M.) for six samples, each sample composed of pooled tissue of six animals. *: statistically significant; p < 0.05 for each time point compared to time 0.

The implant-dependent decrease in MVEM phospholipase A₂ activity was even more marked in the tissue adjacent to nonbonding implants; there was no MVEM phospholipase A₂ activity in the contralateral limb. MVEM alkaline phosphatase specific activity became only slightly elevated during healing, but no differences in magnitude between treatment and control limbs were evident. Matrix vesicle density was either comparable to that of normal healing bone or was depressed. These data demonstrate that there is both local and systemic regulation involved.

Role of Matrix Vesicle Components in Mineral Deposition

The data presented above show that changes in alkaline phosphatase or phospholipase A₂ activities can be correlated with altered matrix vesicle function in vivo. Other components of the vesicle are now known to play a role in the calcification process. Most information is

the onset of calcification in the tissue and may reflect its role in the initiation of hydroxyapatite formation as a constituent of matrix vesicle proteolipids and CPLX.

Matrix vesicle morphometrics and MVEM biochemical parameters are sensitive to regulation in vivo. When bone-bonding and non-bonding ceramic and metal implants were inserted into the marrow-ablated tibia, changes were observed in the normal healing pattern, both in the treated leg and in MVEM isolated from the contralateral limb [7, 44, 53]. Bone-bonding materials stimulated MVEM alkaline phosphatase specific activity and increased matrix vesicle density in the treated limb at early time points. While MVEM alkaline phosphatase behaved like that seen in normal healing at later times, matrix vesicle concentration (/µm²) was decreased with respect to normal. MVEM phospholipase A₂ specific activity was lower in tissue adjacent to the bone-bonding implants and was markedly inhibited in the contralateral limb.
derived from studies of cartilage and cartilage cells. Initial sites of hydroxyapatite deposition are observed on the inner vesicle membranes [1-3, 9]. CPLX and proteolipids, shown to support calcification in vitro [16, 17, 23, 50], have been isolated from matrix vesicles [23, 73], suggesting that they could function as mineralization initiation sites. Other observations support this hypothesis. CPLX is present at sites where mineralization is about to occur or has just started, as evidenced in hypertrophic cartilage, metaphyseal bone, fracture callus, and following induction of heterotopic bone formation [10, 16-20]. Proteolipids are enriched in matrix vesicles and participate in formation of CPLX, in part by structuring phosphatidylserine [21, 22]. Proteolipids may also act as ionophores, exporting protons and importing calcium and phosphate [27, 68].

Enzymes like alkaline phosphatase, pyrophosphatase, ATPase, phospholipase A₂, and NTP-pyrophosphohydrolase, which exhibit increased activity as the growth plate mineralizes, have been localized to matrix vesicles [32, 33, 38, 70] and are regulated by factors which monitor calcification [24, 55, 56, 58]. Robison [52] first observed the relationship between bone formation and increased alkaline phosphatase and postulated a role in raising local concentrations of Pᵢ [9, 40]. It is possible that more than one matrix vesicle alkaline phosphatase exists [11], each with a distinct function.

Other matrix vesicle proteins appear to play a role in calcification. Uptake of mineral ions by isolated matrix vesicles incubated in synthetic cartilage lymph has been shown to follow a consistent pattern. After an initial lag, vesicles rapidly accumulated Ca²⁺ and Pᵢ, an ability readily destroyed by proteases. A subset of EGTA-extractable proteins selectively bound Ca²⁺ in the presence of lipids and cross-reacted with antibodies to calpain II, suggesting that this protein was involved in ion uptake [34].

Matrix maturation is important in the cascade of extracellular events which lead to bulk phase hydroxyapatite formation. Mineralization of the matrix involves proteoglycan degradation [26, 32, 41, 42], including aggregate breakdown, proteolysis of the core protein, and glycosaminoglycan degradation [48, 49]. Cytochemical studies [71] suggest that glycosaminoglycan loss is mediated by matrix vesicles. Proteases are present in matrix vesicles [29, 38], suggesting that they participate in matrix processing. Their distribution is cell maturation-dependent; neutral and acid metalloprotease activity is highest in matrix vesicles produced by growth zone chondrocytes [29]. Since a significant percentage of the metalloproteases are in active form, breakdown of the matrix vesicle may be the event which releases these enzymes for matrix maturation and subsequent calcification.

Regulation of Matrix Vesicle Mineral Formation

Membrane phospholipids are subject to regulation by a number of enzymes; thus, their metabolism can influence the structure and function of matrix vesicles [39, 64-66]. Phospholipase A₂ activity increases commensurate with maturation of the growth plate [70]. In matrix vesicles isolated from cultured chondrocytes, enzyme activity is higher in growth vesicles produced by growth zone cells than in vesicles produced by resting zone cells [55]. This enzyme catalyzes the rate-limiting step in prostaglandin production [13, 36], and is also required for fatty acid turnover, resulting in changes in membrane fluidity. This can have consequences for ion transport and enzyme activity [46, 51]. The fluidity of the membrane can alter its structural characteristics [31] ultimately changing the ability to support mineralization. Another role for phospholipases in vesicles may be to degrade membrane phospholipids, resulting in loss of vesicle integrity [67, 72]. Those lipids which have formed mineral complexes appear to be resistant to the action of phospholipases [50]. This may explain the difference in susceptibility to hydrolysis observed when phospholipids isolated from plasma membranes or matrix vesicles are incubated with exogenous phospholipases [58].

Other matrix vesicle enzymes may play roles in removal of mineralization inhibitors [32]. ATP and other nucleotide triphosphates are present in matrix vesicles and inhibit crystal growth. Their hydrolysis by NTP-pyrophosphohydrolase [32] generates pyrophosphate, another polyphosphate inhibitor, subsequently degraded by pyrophosphatase. As hydroxyapatite crystals grow, protons are released, potentially lowering local pH. One function of matrix vesicle carbonic anhydrase may be to prevent this acidification. Other proteins are present, such as actin [45] but probably do not have a role in mineral deposition.

The suggested chain of events and their localization in the extracellular matrix, on the vesicular membrane, and within its lumen support the current hypothesis on the active role of matrix vesicles in the initiation of mineralization (Fig. 3).

References

Matrix Vesicles

Current Hypothesis on Matrix Vesicle Regulation

**Figure 3.** Current hypothesis on matrix vesicle regulation. This diagram demonstrates a possible chain of events and their suggested localization regulating the process of initial calcification within extracellular matrix vesicles, the data are taken from literature cited in the text.

Matrix Vesicles

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Discussion with Reviewers

Reviewer I: Matrix vesicles are extracellular - how can they keep calcification under cell control?

Authors: Recent studies have shown that matrix vesicles are under autocrine regulation by chondrocytes. These cells produce vitamin D metabolites which have direct, non-genomic effects on the matrix vesicle membrane. In effect, the cell is able to regulate events in the matrix by remote control. While matrix vesicles and plasma membranes, the effects are seen only in the matrix vesicles and not in the plasma membranes isolated from intact cells incubated with hormone. These data suggest that the chondrocyte can down regulate any effects on the cell, whereas, the matrix vesicle is unable to do so. In this manner, the cell is able to synthesize the matrix vesicle under genomic control and then regulate its activity later by non-genomic mechanisms.

T.A. Einhorn: The authors note that, in addition to their role in mineralization, matrix vesicles may be involved in various biochemical events which take place during the degradation of proteoglycan aggregates. These proteolytic activities are presumed to be necessary in order to create the necessary biochemical environment such that mineralization may proceed. Have the authors considered the possibility that these matrix-vesicle associated proteases may also be involved in the binding or activation of growth factors or cytokines?

Authors: Dr. Einhorn raises an interesting point. Preliminary data from our laboratory suggest that matrix vesicle enzymes do play a role in activation of cytokines in the matrix.

T.A. Einhorn: The authors imply that the lipid content of matrix vesicles is distinct from the cell in general and the plasma membrane. If this is true, what is the origin of the matrix vesicle in relation to the cell? How does it form? Is its trilaminar membrane similar to that found in chondrocytes or osteoblasts or is it unique to the matrix vesicle?

Authors: The phospholipid composition of matrix vesicles differs quantitatively from that of the plasma membrane from which it is derived. This may be due to formation of specific phospholipid domains prior to the release of the matrix vesicle from the plasma membrane. Another mechanism may be phospholipid metabolism in the matrix vesicle itself.

T.A. Einhorn: Various investigators have suggested that matrix vesicles are nothing other than artifacts of tissue preparation. What evidence do the authors have that this is not the case and that matrix vesicles are true physiological entities?

Authors: In early descriptions of matrix vesicles they were compared biochemically to the chondrocyte pellet. Not surprisingly, they exhibited enrichment of alkaline phosphatase since they represented a relatively pure population of membranes and the plasma membrane alkaline phosphatase of the cell was diluted by other cellular constituents. This may have led to some of the confusion concerning the concept of matrix vesicles as "dynamic cell debris" versus a legitimate extracellular organelle. In our studies, matrix vesicles are compared to purified plasma membranes. Because they are extracellular, homogenization of the tissue is not needed for their isolation following enzymatic degradation of the extracellular matrix. They can be easily separated from the cells by gently pelleting the latter. The cells are more than 95% viable by trypan blue dye exclusion suggesting that fragmentation of the plasma membrane is minimal. The matrix vesicles isolated in this manner are "right side out." It is necessary to homogenize the cells in order to purify the plasma membranes. When the plasma membranes are isolated, they may be inside out or right side out. If matrix vesicles were fragments of the plasma membrane, they would also be one-half inside out and one-half outside out and alkaline phosphatase activity would equal that of the plasma membrane.
Matrix Vesicles

If enrichment of enzyme activity were only two-fold, positive identification would still not be possible, since the vesicles could have been formed by trypsinization and be right side out. For this reason, the alkaline phosphatase specific activity of the matrix vesicles must be more than twice that of the plasma membranes in order for them to be considered by us to be "true physiological entities." In many of our preparations, the alkaline phosphatase specific activity of the matrix vesicles is ten times that of the plasma membranes.

This quality control is not possible when using matrix vesicle-enriched microsomes prepared from homogenized tissue. In order to isolate the purest matrix vesicles possible, both differential and density gradient centrifugation are used. The matrix vesicles that are obtained using this methodology are comparable to the ones that are obtained using the methods described above. While it is not possible to rule out that we have merely isolated an alkaline phosphatase-rich domain of the plasma membrane, the similarity of the composition of matrix vesicle-enriched microsomes with the matrix vesicles isolated from enzymatically digested tissue, argues that we have not.

J.M. Shapiro: It is difficult to understand why the authors utilize a high speed fraction (MVEM), derived from the tissue homogenate as a substitute for native matrix vesicles. The homogenate would contain many cell types and, therefore, many different kind of vesicles would be generated. Moreover, as the MVEM which is mainly endoplasmic reticulum, it would have a different lipid and protein composition from plasma membrane, which in turn is different from that of the matrix vesicle. Would it not be better to use either a plasma membrane fraction or isolated vesicles for the studies described in the manuscript?

Authors: We agree that the MVEM are not the fraction of choice if one wants to study matrix vesicles. We used MVEM because the tissues from which we isolated them were frozen to permit transfer from Israel to the USA. The result was freeze/thawed tissue, itself somewhat homogenized, and, therefore, classical matrix vesicle isolation techniques were precluded. A further complication was the difference in consistency in the tissues with time. Homogenization eliminated some of the variation. Some confusion may result from the term "microsomes," which to many biochemists implies rough or smooth endoplasmic reticulum. Certainly rough endoplasmic reticulum can be ruled out, as there is no RNA present in the matrix vesicle preparation. Microsomes can also refer to any membrane preparation that forms vesicles under aqueous conditions. We are confident that the MVEM were a highly enriched fraction and that endoplasmic reticulum contamination, or of any other subcellular membrane organelle, was quite low, based on marker enzyme studies.

We chose not to use the plasma membrane fraction because other studies in our laboratory have shown that the plasma membrane is regulated in a differential manner from the matrix vesicles, and it was matrix vesicles we were interested in based on our cytomorphometric studies.

J.M. Shapiro: While it is very interesting that bone cells secrete matrix vesicles and mineral is present in the vesicles at sites of repair, mineral is also seen in collagen. Would the authors please provide some information on the relationship between matrix vesicle and collagen mineralization at these repair sites? In addition, are values for matrix vesicle related to values obtained for other components of the matrix?

Authors: Whether or not the mineral present in matrix vesicles is directly related to mineral on the collagen fibrils has not been satisfactorily resolved. It is our hypothesis that the mineral within the vesicle is involved in its breakdown and the subsequent release of proteases needed to modify the matrix for bulk phase mineralization. At the transmission electron microscopy level, it appears that at least some of the mineral present in the matrix vesicles contributes to calcospherite formation.

J.M. Shapiro: The data on phospholipase activities at mineralizing sites is of considerable interest. Why was there inhibition of phospholipase activity in tissue close to implants, etc.? Does this imply that there exists in the extracellular matrix a local phospholipase inhibitor; alternatively, does the implant modulate osteoblast endoplasmic reticulum enzyme activity (MVEM) and this results in modification in matrix vesicle lipase activity?

Authors: We are also intrigued by the regulation of phospholipase A_2. We are presently examining the effects of specific phospholipase A_2 activators and inhibitors to try to dissect the problem more effectively.