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THE ROLE OF MATRIX VESICLES IN GROWTH PLATE DEVELOPMENT AND BIOMINERALIZATION

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1. ABSTRACT

Skeletal cells control the initiation of mineralization *in vivo* and determine the selective distribution pattern of mineralization by releasing calcification-initiating, submicroscopic, extracellular matrix vesicles (MVs) at selected sites in the extracellular matrix. The overall objective of this review is to outline what is currently known about the mechanisms of MV biogenesis and mineral initiation, while emphasizing recent observations that enhance our understanding of these mechanisms. Data from studies on the general mechanism of biogenesis of outer membrane vesicles and the formation and function of non-skeletal matrix vesicles is presented to stimulate thought concerning the possible biological functions that these structures may share with MVs.

2. INTRODUCTION

Matrix vesicles (MVs) are ~50-200 nanometers in diameter, extracellular, membrane-invested vesicles, within which the first crystals of calcium hydroxyapatite mineral are generated during biomineralization of growth plate cartilage (Figure 1) (1), newly formed bone (2), tendon (3) and the predentine of teeth (4). Hydroxyapatite crystal formation is usually preceded by an increasing electron density of the MV sap. This increasing density is likely due to the pre-deposition of non-crystalline, precursor $CaPO_4$ mineral.

The growth plate of a growing animal is an opportune site to examine the successive stages of MV biogenesis and calcification because of the remarkable geometric layering of chondrocytes, whose differentiation is synchronized in time as well as in space. Progressively deeper layers of the growth plate show progressively later stages of chondrocyte maturation (Figure 2A). There is an upper layer of proliferating chondrocytes below which are layers illustrating successive stages of chondrocyte differentiation and maturation (upper hypertrophic zone), and finally chondrocyte programmed cell death at the base of the growth plate (in the lower hypertrophic cell zone, sometimes referred to as "calcifying zone"). Matrix vesicles are released in a polarized fashion by budding from the lateral edges of growth plate chondrocytes (Figure 2A and 2B) (5, 6). Polarized budding of MVs also occurs from osteoid-facing surfaces of osteoblasts (2, 7, 8) and from the apical surfaces of odontoblasts (4, 9-11). After polarized release from the cell, MVs soon become encased and immobilized between fibrils of the newly secreted and polymerized extracellular matrix, and then are transported away from the cell with the accumulation of additional secreted matrix proteins. The ability of skeletal cells to polarize vesiculation to specific regions of their outer membrane implies an ability of MV-generating cells to endow the MV precursor membranes with a selected, specific array of proteins, lipids, etc., especially conducive to mineralization. An example of such molecular sorting may be reflected in the selective enrichment in MV precursor membranes of acidic phospholipids (with Ca^{2+}-binding ability) greater than that seen in isolated whole plasma membranes of mother chondrocytes (12). Also, the inventory of major proteins is different in MVs than in chondrocyte plasma membranes, there being a different spectrum of major molecular weights in MVs versus in cell membranes (13) and a relative concentration of alkaline phosphatase and dilution of acid phosphatase in MVs (14).

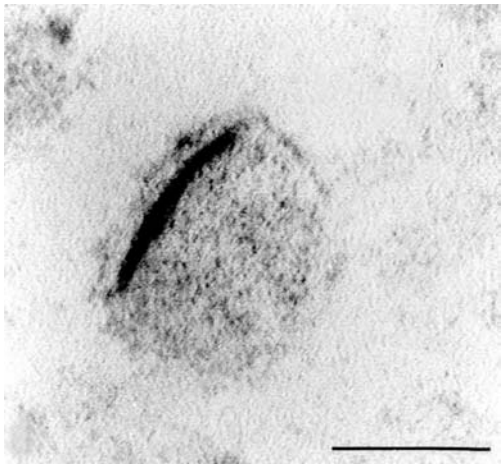

Figure 1. Electron micrograph of a calcifying matrix vesicle in the hypertrophic zone of rat growth plate. The first electron dense crystalline apatite mineral is seen as a needle-like, electron-dense precipitate within the matrix vesicle, often in apposition to the inner leaflet of the vesicle membrane. Stained with lead and uranium x 300,000. Bar=50 nm (Reprinted from "Endocrine Control of Bone and Calcium Metabolism", 8B: 410, Excerpta Medica, Amsterdam, 1984 (177)).

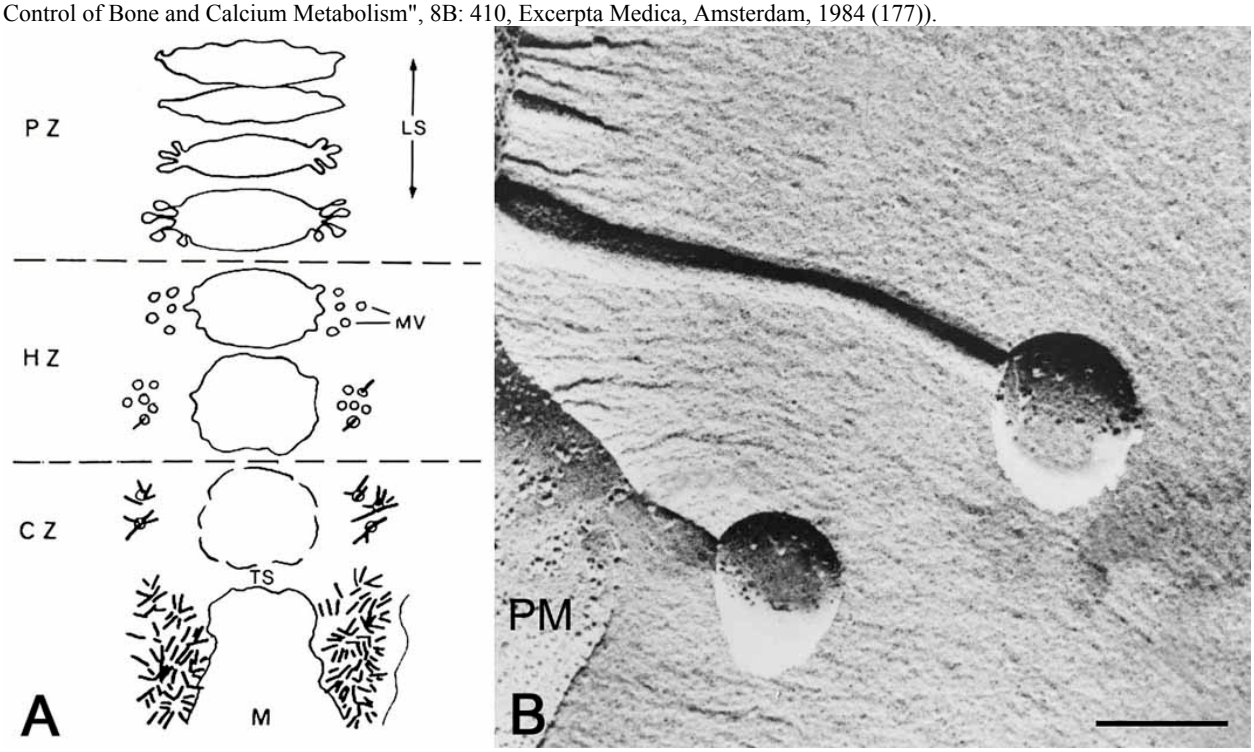

Figure 2. A) Diagram of successive stages of chondrocyte maturation and matrix vesicle budding from the lateral plasma membranes of lower proliferative zone (PZ) and upper hypertrophic zone (HZ) of the growth plate. Needle-like hydroxyapatite crystal deposition begins within MVs of the hypertrophic zone (see Figure 6A & 6B). Lower in the HZ, apatite crystals are released from calcifying MVs. Then, apatite crystals self-nucleate and proliferate to form spherical mineral clusters in the calcified zone (CZ). These apatite clusters grow and ultimately fuse together in the CZ at its junction with the metaphysis (M). Capillaries, growing in from the metaphysis, penetrate the unmineralized transverse cartilage matrix septa (TS), while unresorbed, fully mineralized longitudinal septa project into the metaphysis, where they will serve as a scaffolding for the deposition of new bone matrix by in-growing osteoblasts. B) Matrix vesicles bud from the tips of villi of the hypertrophic chondrocyte plasma membranes (PM). This freeze-fracture replica also illustrates the many small intramembranous particles that lie within the protoplasmic face of the chondrocyte plasma membrane. x 89,000. Bar=0.2 micrometer Reprinted from Cecil, *et al.* Metab. Bone Dis. & Rel. Res. 1: 89, 1978 (5)).

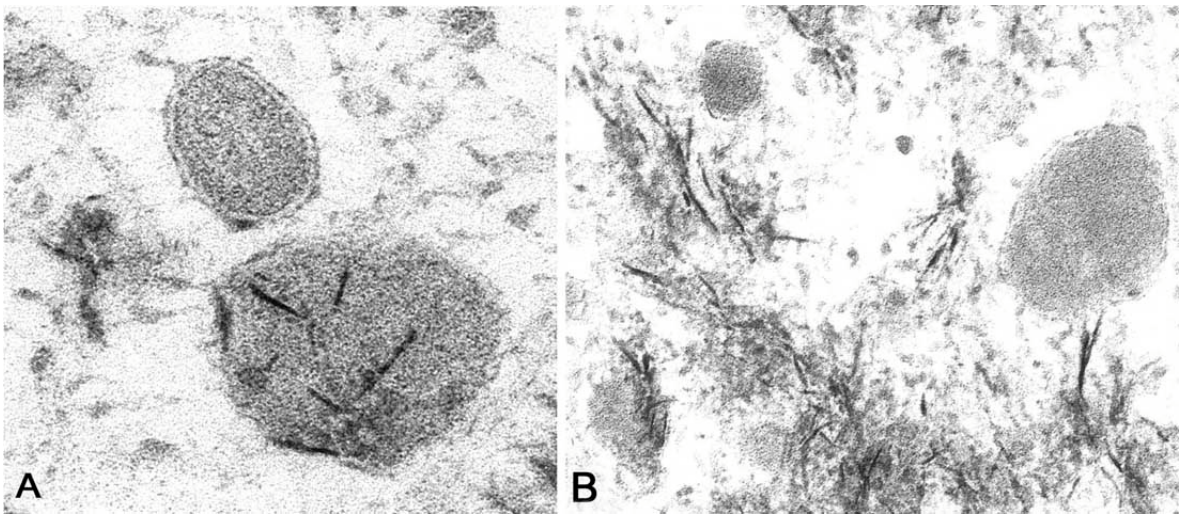

Figure 3. A) Transmission EM shows early accumulation of needle-like apatite crystals within the sap of MVs in growth plate cartilage matrix. (x 250,000). B) Transmission EM of more advanced MV calcification in lower hypertrophic zone matrix, showing accumulation of peri-vesicular clusters of needle-like apatite crystals (x 100,000).

The basic conclusion to be drawn from evidence of cell polarity in MV biogenesis is that the cells can control the molecular composition of budded membranes, and probably do so in order to achieve an optimal level of calcifying activity at a cell-determined, non-random site in the adjacent matrix.

Mineral crystals in MVs increase in number as the distance between individual MVs and the upwardly advancing mineralization front decreases (Fig 3A). The latter is composed mostly of extravesicular mineral crystals (Figure 3B).

3. MOLECULAR COMPONENTS OF MATRIX VESICLES

3.1. Lipids

The membranes of isolated MVs constitute a lipid bilayer, similar in structure to that of the outer plasma membrane of the mother cell. As such, the MV membranes were shown by lipid analysis to be enriched in typical plasma membrane phospholipids (cholesterol and sphingomyelin), but also to contain an unusual concentration of acidic phospholipids (e.g. phosphatidylserine and phosphatidic acid) (12). The acidic phospholipids may serve as a non-energy-requiring calcium trap during mineralization (12, 15, 16).

3.2. Alkaline phosphatase

The enzyme alkaline phosphatase (ALP), also known as "tissue non-specific alkaline phosphatase" (TNAP), is the ALP isoform that is most concentrated in bone, liver and kidney, and is characteristically enriched in MVs, i.e. about ten-fold over the specific activity of ALP in whole isolated chondrocytes (17). Alkaline phosphatase is concentrated at the outer surfaces of the MV membrane (Figure 4) where it is anchored to glycosylphosphatidylinositol (GPI) of the membrane lipid bilayer (6, 18, 19). The membrane association of ALP is essential for calcium deposition by isolated matrix vesicles (18, 20). If ALP is released from MVs by mild detergent treatment, leaving the MV membranes intact, then the ability of isolated MVs to deposit $CaPO_4$ is reduced (20). Also, there is an increasing activity of ALP at MV membrane surfaces as the vesicles near the calcification front of the growth plate (19).

The enzyme alkaline phosphatase was first identified by Robison in 1923, who also was the first to suggest that ALP activity in bone stimulates $CaPO_4$ mineral deposition (21). Although much debate and skepticism regarding Robison's hypothesis persisted over the next sixty years, the balance of current evidence, strongly supported by recent studies of the hereditary disease hypophosphatasia (22), indicates that the alkaline phosphatase isozyme that is concentrated in bone (TNAP) is essential for normal mineralization. Hypophosphatasia in children is a heritable condition characterized by deficient expression of the TNAP isoenzyme of ALP. This deficiency results in severe hypomineralization of growth plates and bones (23). The presence of skeletal hypomineralization was confirmed in mice lacking the Akp2 gene for TNAP (24). In addition to hypophosphatasia serving as evidence of a positive role for ALP in mineralization, several other experimental approaches have confirmed the importance of ALP in promoting calcification of cartilage and bone (25).

3.3. Other phosphatases that contribute to matrix vesicle mineralization

Adenosine monophosphoesterase (AMPase) is another non-ALP phosphatase that is enriched in MVs (17). More recently, it has been shown that adenosine monophosphate (AMP) is one of the most effective substrates that can be added to preparations of isolated MVs in order to augment $CaPO_4$ deposition. It was recently shown, using a newly developed ^{40}Ca assay (26), that AMP supports crystalline $CaPO_4$ deposition more

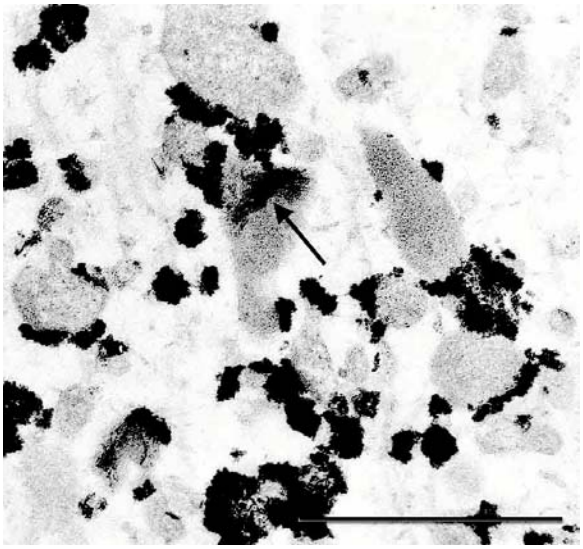

Figure 4. Matrix vesicles of hypertrophic growth plate cartilage, stained for alkaline phosphatase. The black granular alkaline phosphatase reaction product is seen mostly at the surfaces of the investing membranes of the matrix vesicles in this electron microscopic cytochemical preparation. Needle-like profiles of apatite mineral (indicated by an arrow) are seen within one MV. (x 95,000. Bar=0.5 micrometer Reprinted from Matsuzawa and Anderson, J. Histochem. Cytochem. 19:801, 1971 (19)).

effectively than does ATP, when either is supplied as substrate for $CaPO_4$ deposition by isolated matrix vesicles (27). ALP, as well as AMPase, can hydrolyze AMP, thus releasing inorganic phosphate (Pi) for incorporation into nascent $CaPO_4$ mineral. At present, the relative contribution of Pi from hydrolysis of AMP by AMPase versus by ALP is not known. It is, nevertheless, likely that AMPase makes a significant contribution of Pi to mineral formation, since some mineralization of growth plate and bone persists in hypophosphatasia, despite the complete loss of ALP enzymatic activity (24, 28).

Inorganic pyrophosphatase (PPiase) was shown to be relatively concentrated in preparations of isolated MVs by Ali, *et al* (17). This phosphatase also may play a role in promoting MV mineralization. Not only is PPiase capable of hydrolyzing inorganic pyrophosphate (PPi), thus neutralizing the inhibitory effect of PPi on hydroxyapatite mineral crystal formation (29). Inorganic pyrophosphatase activity also hydrolyzes PPi, yielding two Pi molecules for incorporation into nascent $CaPO_4$ mineral. PPi concentration variations in the skeletal extracellular fluid have a bimodal effect on mineralization. Lower extracellular PPi concentrations, up to 1 mM, stimulate mineralization, because most PPi is hydrolyzed by TNAP and other pyrophosphatases to yield Pi for incorporation into nascent mineral, while with PPi concentrations above 1-2 mM, the excess PPi is not hydrolyzed by endogenous pyrophosphatases, thus leaving unhydrolyzed PPi with the capability of inhibiting mineralization (Figure 5A and B). This has been confirmed in organ cultures of fetal chick long bones, where addition of up to 1 mM PPi to the culture medium promoted mineralization, while PPi concentrations above 1 mM tended to retard mineralization (2). More recently, deletion of the gene for nucleoside triphosphate pyrophosphohydrolase (NPP1) in mice, an enzyme that hydrolyzes ATP to yield PPi, led to diminished mineralization in long bones, which was even more severe when the genes for both ALP (Akp2) and NPP1 (Enpp1) were deleted (28). As with the hydrolysis of AMP, the hydrolysis of PPi to yield Pi for mineral formation is accomplished in MVs by both inorganic PPiase as well as by TNAP, and, at present, the relative contribution of PPiase versus TNAP in yielding Pi for *in vivo* bone mineral formation is not known. Recently, the dileucine motif of NPP1 was identified as a molecular targeting motif for concentrating PPi-generating NPP1 at plasma membrane sites of osteoblasts from where MVs bud off (30).

Matrix vesicles are also enriched in ATPases (17, 19), including Ca^{2+} ATPase (7). Considerable evidence has been presented showing that hydrolysis of ATP, by the ATPases in isolated MVs, significantly enhances $CaPO_4$ deposition *in vitro* (31). However, the hypothesis that ATPase activity is the most important contributor of Pi for the formation of nascent $CaPO_4$ mineral is weakened by several prior observations. First, a significant proportion of the ATPase activity in isolated MVs is in the form of ATP triphosphate pyrophosphohydrolase (equivalent to the NPP1 enzyme described above) whose major ATP hydrolysis product is PPi (32, 33), and PPi in excess has the ability to inhibit crystalline $CaPO_4$ mineral deposition (29). Second, as indicated above, adding AMP is even more effective than adding ATP to promote the calcification of isolated matrix vesicles (27). Third, when using ATP as substrate versus AMP, the mineral deposited by isolated MVs is lacking in apatite crystallinity (27), and is composed mostly of non-crystalline CaPPi (34). Thus, it would appear that while ATPase does play a positive role in regulating mineral initiation, its major role may be to provide PPi for hydrolysis by ALP, PPiase, etc., thus yielding Pi for incorporation into nascent mineral.

A new phosphatase, designated Phospho1, has recently been identified and shown to be concentrated in upper hypertrophic zone chondrocytes of the chick growth plate, where mineralization begins in MVs (35). Because of its intense localization in zones of incipient calcification in both growth plate and in developing bone, it has been suggested that Phospho1 activity may release Pi for incorporation into nascent $CaPO_4$ mineral. Although the evidence presented so far in support of a role for Phospho1 in promoting mineralization is indirect, it seems likely that Phospho1 and ALP activities could be synergistic in generating Pi. Also, it will be quite interesting to determine whether Phospho1 is concentrated in calcifiable MVs, as is ALP.

3.4. Non-phosphatase proteins of matrix vesicles that play a role in mineralization

Annexin V is highly concentrated under the membranes of MVs where it appears to function as an inwardly directed Ca^{2+} channel (36-38). Annexin V-stimulated inward transport of Ca^{2+} into MVs could raise

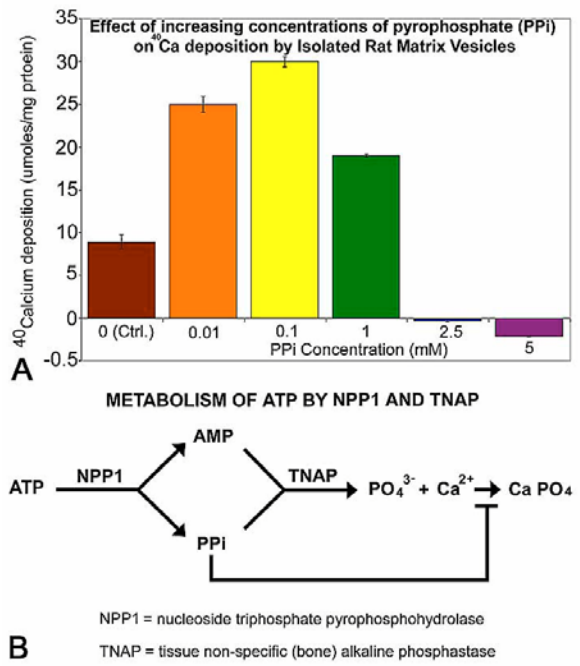

Figure 5. A) Shows the effect of increasing concentrations of Na pyrophosphate added as a phosphatase substrate, upon the amount of $CaPO_4$ deposition by isolated rat MVs. Na pyrophosphate, added at concentrations from .05 to 1.0 mM stimulates $CaPO_4$ deposition above the control level (to which no NaPPi was added). Na pyrophosphate, at concentrations from 2.5 to 5.0 mM, inhibited mineral deposition. B) Diagram outlining the metabolic sequence of events that occurs when ATP is hydrolyzed by nucleoside triphosphate pyrophosphohydrolase (NPP1). Following ATP hydrolysis by NPP1 to yield AMP and pyrophosphate (PPi), both AMP and PPi will be further hydrolyzed to orthophosphate (PO_4^{3-}) by tissue non-specific (bone) alkaline phosphatase (TNAP). The resulting orthophosphate molecules are then incorporated into $CaPO_4$ mineral. Abnormally high levels of non-hydrolyzed PPi (above 1-2mM) would tend to block the formation of $CaPO_4$ mineral (29). Lower endogenous PPi levels, at 1 mM or less, would be more fully hydrolyzed by TNAP and thus would stimulate $CaPO_4$ deposition (2). (Reprinted, with modification, from Anderson, *et al*, Am. J. Path. 164: 841, 2004 (24)).

the intravesicular (Ca^{2+}) x (PO_4^{3-}) ion product, thus promoting initial mineral formation.

Bone sialoprotein (BSP), osteonectin (ON) and osteocalcin (OC) are Ca-binding non-collagenous matrix proteins of bone, which have been shown to be concentrated in isolated rat growth plate MVs by Western blotting (39). Of the three, only BSP has been shown to promote *in vitro* mineralization (40). Neither ON, nor OC appear to promote bone mineralization. Recent studies in which the mineralization of bone and soft tissue was analyzed in transgenic mice deficient in ON showed that the bones had higher than normal mineral content (41), suggesting a mineral inhibiting function for ON *in vivo* (41). Osteocalcin-deficient transgenic mice exhibit an accelerated rate of bone formation without changes in mineral content of the increased new bone that is formed (42, 43). Thus, of the three Ca-binding non-collagenous bone matrix proteins that have been identified thus far in MVs, i.e. BSP, ON and OC (39), only BSP would appear to play a positive role in mineral initiation by MVs.

As yet undefined protein(s) located in the MV membrane constitute the sodium-dependent phosphate transporter that promotes ingress of Pi into MVs at an early stage of mineral initiation (44, 45). This Na-dependent PO_4 transporter is quite distinct from the ALP of MVs. It has an earlier timing of its peak activity, and its relative insensitivity to the rather specific ALP inhibitor levamisole (44). Thus, it has been suggested that the Na-dependent PO_4 transporter plays a role in early phases of ion accumulation in MVs, while ALP would be important for a later phase of MV mineral initiation. This suggestion is consistent with recent observations on the role of MVs in the hypomineralization that is seen in hypophosphatasia (23, 24), where TNAP deficiency does not inhibit the early accumulation of apatitic mineral within matrix vesicles. Instead, TNAP deficiency appears to inhibit the propagation phase of mineralization into the extravesicular matrix of growth plate cartilage and metaphyseal bone (23, 24).

4. MECHANISM OF MATRIX VESICLE CALCIFICATION

It would appear that mineralization, as initiated by MVs, is essentially a biphasic phenomenon. Phase 1 is concerned with the formation of the first crystals of mineral within MVs (Figure 6A). Phase 1 probably begins with the absorption of Ca^{2+} into the MV membranes and then into the MV sap, due to a local enrichment of Ca-binding phospholipids and proteins in MVs (12, 16, 36, 46, 47), plus the inward Ca^{2+} transporting activity of Annexin V. Inward Ca^{2+} transport would be accompanied by PO_4^{3-} accumulation within the MV, as augmented by the PO_4-concentrating activities of the Na-PO_4 transporter (44, 45) plus the enzymatic activity of Phospho1, which appears to play an early role in PO_4 concentration by MVs (35). Alkaline phosphatase activity also may function in the early accumulation of PO_4 within MVs, but seems to be more important in providing an enrichment of PO_4 at the perimeter of MVs during early, Phase 2 mineral propagation (24). In all likelihood, Ca^{2+} and PO_4^{3-} accumulation within MVs overlaps to some degree. When sufficient Ca^{2+} and PO_4^{3-} have accumulated within the MVs, $CaPO_4$ mineral will begin to precipitate. The first $CaPO_4$ deposited is a non-crystalline form, i.e. amorphous $CaPO_4$ (ACP) (48, 49). ACP is thought to convert to octacalcium phosphate, whose crystals are then transformed into the highly insoluble hydroxyapatite (1, 49, 50). Hydroxyapatite crystals must penetrate the MV membrane and be exposed to the extracellular fluid in order to initiate phase 2 (Figure 6B). Matrix vesicle membrane breakdown may be assisted by the hydrolytic action of phospholipases (51) and proteases that have been identified in MVs (52-54). Phase 1 of mineral initiation thus reflects a cascade of multiple molecular interactions that occur

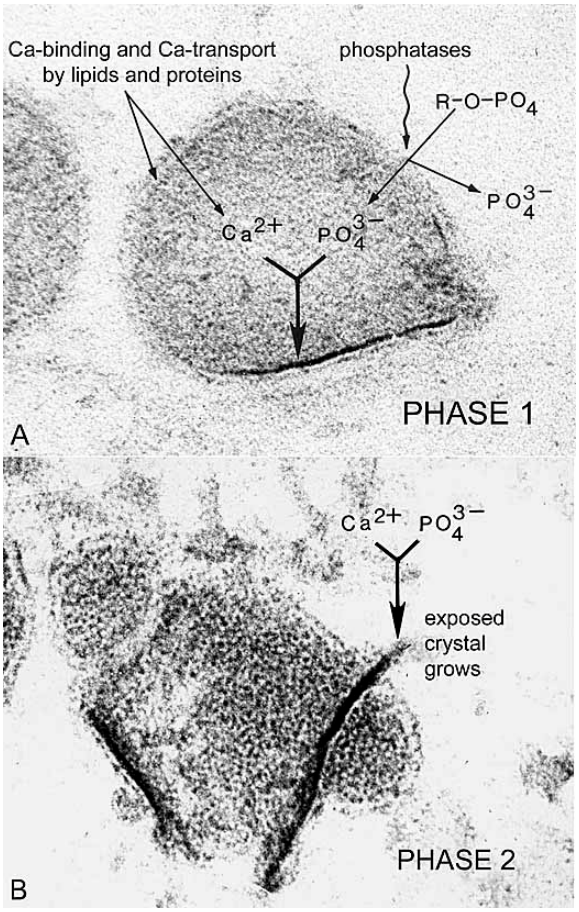

Figure 6. A) Scheme for mineralization in matrix vesicles. During Phase I, intravesicular calcium concentration is increased by its affinity for lipids and Ca-binding proteins of the vesicle membrane interior. Phosphatase, for example alkaline phosphatase, pyrophosphatase, or adenosine triphosphatase at the vesicle membrane, acts on ester phosphate of matrix or vesicle fluid to produce a local increase in PO_4 in the vicinity of the vesicle membrane. The intravesicular ionic product (Ca^{2+}) x (PO_4^{3-}) is thereby raised, resulting in initial deposition of $CaPO_4$ near the membrane. (Reprinted with modification from Anderson, Metab. Bone Dis. 1:84, 1978 (178)). B) With mineral crystal accumulation and growth, intravesicular crystals are exposed to the extravesicular environment. Phase 2 begins with exposure of preformed apatite crystals to extravesicular fluid, which in normal animals is supersaturated with respect to apatite, enabling further crystal nucleation to take place. Matrix vesicles pictured are in rat growth plate cartilage. (Reprinted from Anderson, Metab. Bone Dis. 1:83, 1978 (178)).

within MVs because of their unique molecular composition and 3-dimensional membrane structure.

Phase 2 (Figure 6B) begins with crystal penetration of the MV membrane, thus exposing preformed mineral crystals to the extracellular fluid. The rate of mineral crystal proliferation will be governed by extravesicular fluid conditions such as the levels of ionic Ca^{2+} and PO_4^{3-} in the extracellular fluid at the site of mineral initiation, the pH of the extracellular fluid, and the presence of molecules in the extracellular fluid that can control the rate of mineral crystal propagation (55). Included in this group of extravesicular mineralization-regulating molecules are: 1) collagen types I and II, both of which can promote mineral proliferation (56-58); 2) Ca-binding matrix proteins including BSP, which has the ability to promote mineralization (40) while ON and OC may retard mineralization (41, 43); 3) acidic Ca-binding proteoglycans of the extracellular matrix that bind and sequester ionic Ca^{2+}, thus inhibiting mineral propagation into the growth plate cartilage matrix (59-61); and 4) the local concentration of extravesicular phosphoester substrates for MV phosphatases, including ATP, AMP and PPi (24, 28). As discussed above, all three of these phosphoesters are hydrolyzed to yield Pi for incorporation into propagating mineral by the enzymatic activity of ALP, which is attached to the outer surfaces of the MV membrane. An over-abundance of extracellular PPi would tend to inhibit mineral propagation, while physiologic levels of PPi, in the micromolar range, would tend to promote mineralization (2, 24, 28).

Phase 2 mineral propagation continues with the accumulation of new self-nucleated crystals of hydroxyapatite to form spherical clusters at the surfaces of MVs (Figure 3B). This spherical pattern of early mineral accumulation around MVs in growth plates (1) is also seen in newly formed bone (2), in dentine (4, 9-11), and in calcifying turkey tendon (3, 57). After a short period, the proliferating extra-vesicular crystals will come into contact with collagen fibrils of the surrounding matrix, at which point collagen would begin to play a major role in nucleating and orienting newly formed apatite crystals (3, 57). In the growth plate, the transfer of mineral-nucleating activity from MVs to collagen is facilitated by matrix proteins, which provide a molecular bridge between MVs and collagen type II fibrils (56). These bridging proteins include proteoglycan link protein and proteoglycan hydroxyapatite binding region (58). Crystals that are generated within and at the surfaces of collagen fibrils form in coalignment with the typical 64 nanometer axial periodicity of the collagen fibrils (62). Thus, there is persuasive experimental evidence indicating that MVs and type I or II collagen fibrils work synergistically to achieve the full mineralization of cartilage and bone matrix.

Early on, it had been suggested that type X collagen might promote phase 2 calcification in the growth plate, after it was discovered that the distribution of type X collagen is restricted to the hypertrophic zone, where most calcification occurs (63). However, most recent studies on the effect of a dominant negative mutation of the type X collagen gene do not show a significant defect in the process of bone mineralization (64). Thus, the balance of present evidence appears not to support a major role for type X collagen promoting skeletal mineralization.

5. NEW EVIDENCE OF NON-MINERALIZING FUNCTIONS OF MATRIX VESICLES

5.1. Bone morphogenetic proteins

BMPs have recently been identified in MVs in significant quantities by western blots, using proteins from

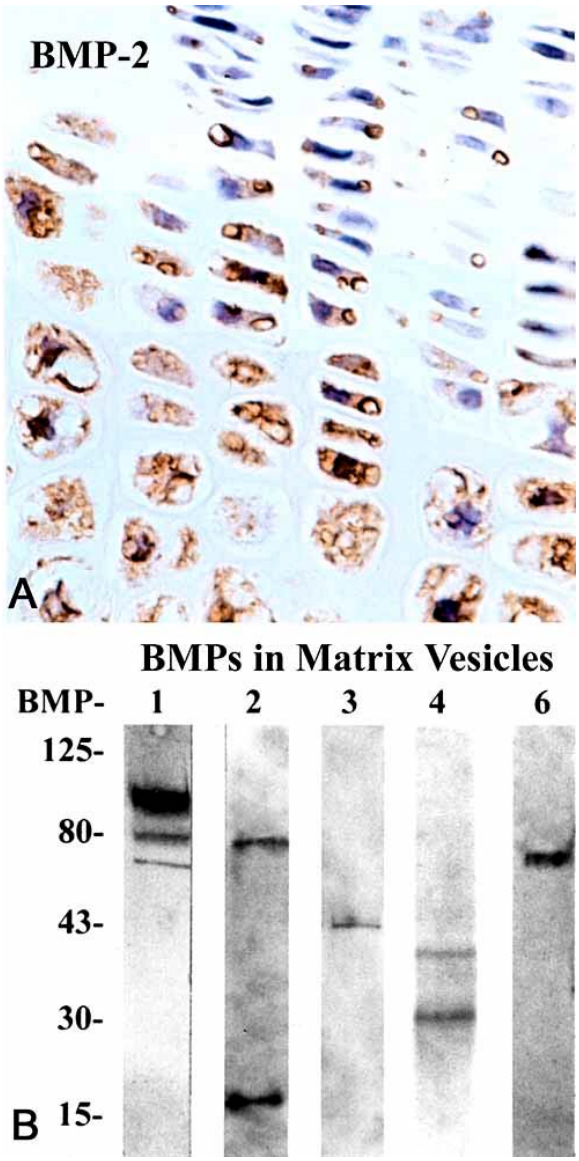

Figure 7. A) Rat growth plate, immunostained for BMP-2, showing most brown-staining BMP-2 in the cytoplasm of maturing chondrocytes of the upper hypertrophic zone. This is the level in the growth plate where most MV budding occurs. (x 1,250. Reprinted with permission from Anderson, *et al.*, J. Histochem. Cytochem. 48: 1493, 2000 (67)) B) Bone morphogenetic proteins (BMPs-1, 2, 3, 4, 5 and 6 are detectible in isolated rat growth plate MVs by Western immunoblots. BMP-7 was not detected.

isolated rat growth plate MVs (39). The BMPs are a group of structurally related, 15 to 22 KD proteins that possess the remarkable ability to induce ectopic bone formation when implanted subcutaneously into immunocompromised animals (65, 66). It is likely that MVs receive BMP proteins from the cytoplasm of upper hypertrophic zone chondrocytes at the time of MV budding, since MV generation is maximal at this level (1). The upper hypertrophic zone chondrocytes have been shown to express and contain the highest levels of BMP-1, 2, 3, 4 and 6 in the growth plate (Figure 7A) (67). Although the finding of BMPs in growth plate MVs (Figure 7B) is preliminary and needs to be confirmed, it has important implications because it suggests that MVs of the growth plate may function as carriers of important morphogenetic information delivered to the subjacent metaphysis where new bone is forming. This finding is reminiscent of the suggestion advanced several years ago by Slavkin, *et al* (1972), who proposed that MVs may function as carriers of morphogenetic signals during tooth development (11). Thus, in the case of the growth plate, MVs also may function as carriers of morphogenetic signals, such as BMPs, that are involved in signaling uncommitted osteoprogenitor cells subjacent to the growth plate to undergo osseous differentiation. The BMPs carried by MVs to the hypertrophic matrix at the base of the growth plate would be released from the matrix during the vascularization and cartilage matrix resorption that occurs actively at this level (68). BMPs in MVs may in fact stimulate resorption of the growth plate by activating osteoclasts. It has recently been shown that osteoclasts are stimulated by BMP-2 and -4, via BMP receptors on osteoclasts. BMP receptors are also required for osteoclastogenesis (69).

5.2. Matrix metalloproteinases (MMPs) and peptidases

MMPs and peptidases known to be present in matrix vesicles may not only promote mineralization but also regulate bone morphogenesis. Regarding mineralization, it was shown earlier that MVs are enriched in neutral peptidases (53) and metalloproteinases (52). These proteinases have the ability to digest proteoglycans of growth plate cartilage matrix. Studies by Dziewaitkowski and Majznerski (1985) showed quite clearly that aggregates of acidic proteoglycans inhibit calcification during endochondral ossification, more than do proteoglycan monomers (61). Furthermore, it is known that proteoglycans of the growth plate become less aggregated as they approach the calcifying zone of the growth plate (70). Disaggregation and digestion of proteoglycans in the calcifying zone of growth plates is likely due to the release of proteases both from matrix vesicles and from apoptotic chondrocytes, thus promoting phase 2 mineralization.

More recently, it has been shown that MVs isolated from chick growth plate contain active MMPs, and are particularly enriched in MMP-2, -9 and –13 (71, 72). (MMP-2 and 9 are particularly enriched in tumor cell-generated vesicles. See section VI below.) Matrix metalloproteinases –2 and –9 are gelatinases, while MMP-13 is a collagenase that is concentrated in the hypertrophic chondrocytes (73). A null mutation of MMP-9 inhibits apoptosis of hypertrophic chondrocytes, thus causing a five-fold enlargement of the hypertrophic zone of the mouse growth plate (74), plus a failure of vascular invasion from the metaphysis. It is hypothesized that MMP-9 deficiency impedes release and activation of MMP-13 from hypertrophic chondrocytes. MMP-13, which is attached to the surfaces of MV membranes plus MMP-9 work synergistically to facilitate the release and dispersion of vascular endothelial growth factor (VEGF) from terminal,

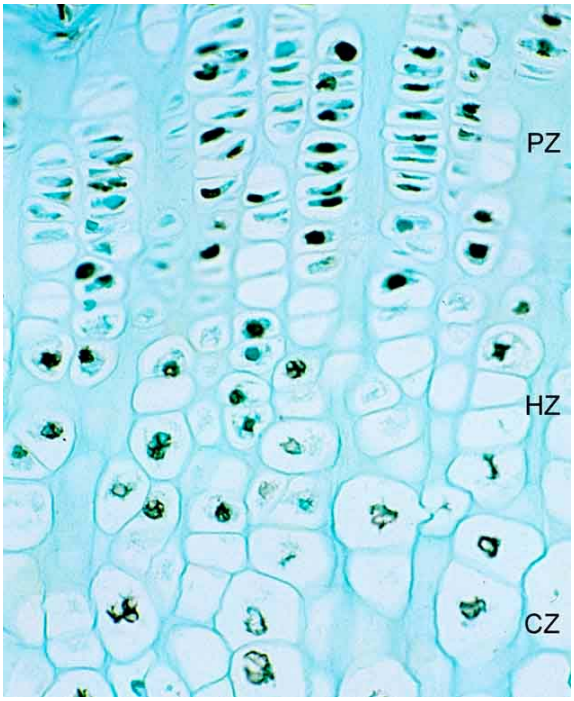

Figure 8. Rat growth plate, TUNEL-stained, shows black-staining nuclei in chondrocytes undergoing programmed cell death (apoptosis). Most hypertrophic zone (HZ) nuclei are in a later stage of apoptosis, with nuclei showing hydropic swelling (oncosis), as well as TUNEL staining. A significant number of lower proliferative zone (PZ) chondrocytes (approximately 1/3) also stain black, indicating that early apoptosis has already begun at this level). (x 1,300).

apoptotic chondrocytes (73). VEGF is an angiogenic factor, highly expressed in hypertrophic chondrocytes, that promotes vascular ingrowth as well as osteoclast recruitment to the base of the growth plate, where active resorption of cartilage matrix precedes the ingrowth of new bone (75-77). MMP-13 is also capable of activating transforming growth factor (TGF-beta), which regulates chondrocyte proliferation and differentiation in the growth plate (78). Thus, MMPs that are concentrated in MVs, especially MMP-9 and -13, working together, appear to have an important role in promoting chondrocyte growth and differentiation, plus vascular invasion, and cartilage matrix resorption, during cartilage replacement by ingrowing bone, at the base of the growth plate.

6. MECHANISM OF MATRIX VESICLE BIOGENESIS

6.1. Relationship of apoptosis to matrix vesicle biogenesis

During maturation of growth plate chondrocytes there is a significant overlap between MV budding from upper hypertrophic chondrocytes (Figure 2A and 2B) and the early phase of chondrocyte programmed cell death as indicated by TUNEL stains of pre-apoptotic chondrocytes (Figure 8). Thus, it has been suggested that the budding of MVs may be an early manifestation of chondrocyte apoptosis, and that the generation of MVs may require growth plate chondrocytes to undergo apoptosis with membrane shedding in order to generate MVs (resembling apoptotic bodies) (79). However, most current evidence seems not to support the hypothesis that MVs are a type of apoptotic body. First, transmission electron microscopic (TEM) studies of growth plate indicate that MVs are already incorporated into newly formed cartilage matrix and have begun to mineralize within the upper hypertrophic zone, above the level in the lower hypertrophic zone where hydropic changes of chondrocyte apoptosis first become notable (1). The characteristic swelling of apoptotic chondrocytes, which reflects leakage of water into the chondrocytes due to a failure cell membrane ion pumps to exclude water, is termed "oncosis" (80). During the swelling process, the endoplasmic reticulum also swells (a process termed "chondroptosis"), but few, structurally typical apoptotic bodies are seen within the perichondrocytic lacunae (81). The final phase of growth plate chondrocyte apoptosis appears to involve shrinkage and condensation of terminal cells at the base of the growth plate, just before vascular invasion occurs from the underlying metaphysis (82). The timing of the terminal phases of chondrocyte apoptosis appear to occur too late to account for the generation of MVs in the upper zone of early hypertrophy. Second, the generation of calcifiable MVs by osteoblasts, in developing bone, and by odontoblasts in pre-dentin has not been shown to be associated with apoptosis in these skeletal cells. Third, very recent reports by Jaovisidha, *et al* (2002) and Kirsch, *et al* (2003) show that induction of apoptosis by sodium nitroprusside in porcine articular cartilage, and by staurosporine in cultured chick tibial growth plate increased apoptosis, but inhibited the generation of calcification-competent MVs (83, 84). In addition, Kirsch, *et al.*, showed that only MVs, but not apoptotic bodies, take up Ca^{2+}, which is accomplished through annexin channels that are not present on apoptotic bodies (84). Thus, although the processes of MV biogenesis and chondrocyte apoptosis tend to overlap in the growth plate (Figure 8), and in articular cartilage, the balance of evidence does not support the hypothesis that calcifiable MVs are true apoptotic bodies.

6.2. Proteins that may play a role in matrix vesicle biogenesis

Now that it has been established that matrix vesicles do not form as apoptotic bodies, the question remains: What is the mechanism of matrix vesicle biogenesis? Unfortunately, there is very little information available on the actual mechanism of MV biogenesis. However, it might be possible to gain some insight by examining the components of matrix vesicles and looking at similar budding processes. The fact that matrix vesicles are not just membrane invested sacs, but contain an actin cytoskeleton (85), suggests that actin regulatory proteins play a role in matrix vesicle biogenesis. In fact, Hale and Wuthier suggest that matrix vesicles form from microvilli and that the retraction of the cytoskeleton is essential for matrix vesicle release (86). It is likely, that along with actin binding proteins, at least one of the small GTPases, i.e.

Rac, Rho, Cdc42, or ARF6, which regulate cortical actin cytoskeletal rearrangements (87-89), will play a role in matrix vesicle biogenesis.

In addition to actin remodeling, membrane rearrangements are likely to be critical for matrix vesicle biogenesis. In fact, membrane rearrangements likely occur, because specific proteins and lipids are selectively enriched in matrix vesicles (12, 16, 90). The concentration of specific lipids may be important for the curvature that the membrane must achieve in order for matrix vesicles to bud from the membrane (91, 92). The concentration of membrane components at specific sites on the plasma membrane, from which matrix vesicles bud, could either be achieved by a shifting and reorganization of the plasma membrane or through the insertion of new membrane or membrane components, at specific sites. It is likely that both of these processes occur. The targeting of new membrane to specific sites on the plasma membrane is probably achieved through Rabs and SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). Rabs are small GTPases, that regulate vesicle trafficking. Over 60 Rabs have been identified, that temporally and spatially regulate membrane traffic (93, 94). One or more of these Rabs may be involved in targeting matrix vesicle components to the correct site on the plasma membrane. A good candidate is Rab3c, which is expressed in osteoblasts and hypertrophic chondrocytes. Like other Rab3 proteins, Rab3c is involved in regulated exocytosis in other cell types (95). SNARE complexes on the other hand are involved in most targeted membrane fusion events (96, 97). The SNARE complex proteins that to date have been found in osteoblasts include VAMP1, syntaxin 4, alpha-SNAP, SNAP23 and SNAP25 (98, 99).

Annexins II, V, and VI are highly concentrated in matrix vesicles (100, 101). In addition to acting as calcium channels that play a role in MV calcification, annexins may play a role in matrix vesicle biogenesis. Annexins have been proposed to play a predominant role in membrane trafficking (102). Their Ca^{2+} binding capabilities allow them to interact with acidic phospholipids (103, 104). Annexins I, II, VI, VII, and VIIIb have been directly implicated in intracellular trafficking, and annexin V is found in caveolae, i.e. membrane invaginations at the plasma membrane (105-113). While the exact mechanisms are unclear, some annexins, including annexin II and VI, are capable of regulating the cortical actin cytoskeleton (114-117). Annexins may function in part by bundling F-actin (116, 117). They could also regulate the actin cytoskeleton by acting as calcium channels, since rises in calcium concentrations can promote the disassembly of actin by activating myosin II, through the Ca(2+)/calmodulin-dependent myosin light-chain kinase (MLCK) (118), or by increasing the affinity of gelsolin, an actin severing and capping protein, to actin (119, 120). In addition, annexin II has been shown to interact with Rac1 complexes (121), and to bind phosphatidylinositol-4,5-bisphosphate (PIP_2) (122, 123), a regulator of actin and membrane rearrangements (124, 125) and a product of activated ARF6 (126). As mentioned above, Rac and ARF6 are key regulators of cortical actin cytoskeletal rearrangements(87-89). Taken together, the ability of annexins to regulate membrane and actin rearrangements and the enrichment of specific annexins in matrix vesicles, suggests that annexins may participate in matrix vesicle biogenesis.

Like matrix vesicle budding, enveloped viruses bud out from the plasma membrane. It is therefore interesting to examine the host proteins that viruses hijack in order to complete their own budding process. One of the main groups of protein complexes that viruses hijack for budding are the ESCRTs (endosomal sorting complexes required for transport) (127-131). These complexes are normally required for the formation of multivesicular bodies (MVBs), through the invagination of endosomal membrane (130). It is interesting to note here, that this invagination process occurs in the opposite orientation as other budding processes inside the cell, and consequently in the same orientation as viral and matrix vesicle budding (i.e. outward from the cytosol). Another host protein that is usurped by some viruses is endophilin 2 (132). In normal cells, endophilin is thought to help promote the curvature of membrane by directly binding and transforming lipid bilayers (133, 134). It could therefore be involved in facilitating the curvature of the plasma membrane required for MV biogenesis.

6.3. Dynamic interaction between calcium, mineralization promoting proteins and lipid rafts

Although, it is likely that MVs originate from specialized domains of plasma membrane that are enriched in mineralization promoting proteins and lipids such as phosphatidylserine, sphingomyelin, and cholesterol, which have a propensity to form lipid rafts (135-137), the exact sequence of events that trigger the clustering of the essential proteins that are required for MV biogenesis, at a specific site of plasma membrane is not completely understood. It is hypothesized that lipid rafts, which represent specialized domains containing GPI-anchored proteins and glycosphingolipids, might serve as a platform for recruitment of mineralization proteins such as GPI-anchored alkaline phosphatase (ALP) and annexins in a calcium-dependent manner. It has been previously shown that increasing the intracellular calcium concentration from 10^{-6} M to 10^{-4} M in isolated growth plate chondrocytes resulted in the release of ALP-enriched MVs (138). It has also been reported that the intracellular calcium concentration of maturing and hypertrophic chondrocytes, (i.e. the site where MV biogenesis and release occurs) is higher than in proliferating chondrocytes (139, 140). An increase in the intracellular calcium concentration might lead to the annexin-driven, formation of lipid rafts as seen in smooth muscle cells (141). Recently, Gillette and Preiss (137), demonstrated that annexin II and alkaline phosphatase, both highly enriched in MVs, were localized in lipid rafts of osteoblasts and that disruption of lipid rafts by cholesterol-sequestering agents inhibited mineralization. Furthermore, they showed that inhibition of expression of annexin II by antisense oligonucleotides reduced mineralization. Thus annexin II might have a role in stabilization of lipid rafts as reported in smooth muscle and mammary epithelial cells (141, 142). Increase in

intracellular calcium concentration also could activate proteinases such as m-calpain (143). Calpains appear to have a role in vesicle formation via degradation of cytoskeletal proteins (144). It has been reported that lower hypertrophic chondrocytes express m-calpain and that inhibition of m-calpain by calpastatin in rat growth plate chondrocyte cultures resulted in suppression of mineralization (145). M-calpain is present in matrix vesicles of MC3T-E1 cells (146). Kifor *et al.* (147), have reported the presence of m-calpain, caveolin-1, and GPI-anchored alkaline phosphatase along with the CaR (Calcium-sensing receptor) fragment, in vesicles isolated from conditioned medium of parathyroid cell cultures, thereby suggesting the association of caveolin-1, GPI-anchored alkaline phosphatase, CaR, and m-calpain-containing micro-domains in MV biogenesis. Other MV enzymes, such as phospholipase A2 (148) and PKC (149) might play a role in MV vesiculation by altering the lipid composition of the plasma membrane and calcium ion flux of growth plate chondrocytes in the presence of 1, 25 dihydroxy -vitamin D3.

7. FUNCTIONAL PLASMA MEMBRANE VESICLES FROM NON-SKELETAL CELLS

The biogenesis of membrane vesicles is not confined to skeletal and dental tissues. Shedding of plasma membrane vesicles, similar in size to matrix vesicles, occurs in various cell types, under normal and pathologic conditions, both *in vivo* and *in vitro* (150-153). These vesicles bud from viable cells at specific sites on the plasma membrane, and can concentrate membrane and cytosolic components (153-156) . Membrane vesicle biogenesis in non-skeletal cells is a process that requires energy and is regulated by extracellular signals (157, 158). There are compositional similarities between matrix vesicles and vesicles shed by non-skeletal cells and it is possible that, at least in part, their function and mechanism of biogenesis is conserved. Shed plasma membrane vesicles from various cell types are therefore worth considering when examining the formation of matrix vesicles.

Of particular interest is the process of vesicle shedding by tumor cells. Vesicles shed by tumor cells have been observed for at least as long as skeletal matrix vesicles (1, 152, 159). Malignant tumor cells shed vesicles at a higher rate than normal cells (155). Like matrix vesicles, vesicles shed by tumor cells, as well as by normal cells, contain proteases. MMP-9, MMP-2, and urokinase plasminogen activator (uPA) have been found in vesicles shed by various tumor cell lines (151, 160, 161). The number of vesicles released and the amount of proteases they contain have been linked to increased invasiveness and metastasis (162, 163).

MMPs associated with shed membrane vesicles have also been implicated in the process of angiogenesis. Taraboletti *et al.* conducted a study involving vesicles shed by endothelial cells and their effect on angiogenesis. They demonstrated the presence of MMP-2, MMP-9 and MT1-MMP as well as the MMP inhibitors, TIMP-1 and TIMP-2. The amount of vesicle-associated MMP-2 and MMP-9 was increased after exposure to the angiogenic factors such as fibroblast growth factor (FGF) and VEGF. They also found that the addition of vesicles containing MMPs to endothelial cells *in vitro*, stimulates endothelial invasion and the formation of capillary-like structures (164). This information suggests that membrane vesicles containing MMPs can regulate angiogenesis. MV-releasing hypertrophic chondrocytes highly express VEGF and VEGF receptor 2/Flk (VEGFR2) and *in vitro* release VEGF into culture media (75, 77). In addition, angiogenesis in the growth plate is mediated by VEGF and MMPs (74, 165). Given that growth plate MVs are known to contain MMPs and their parent cells express VEGF and VEGFR2, it is not a stretch to consider that skeletal MVs may regulate angiogenesis in a similar manner as non-skeletal MVs.

Shed vesicles may also play a role in the regulation of apoptosis. Tumor cells and normal T cells are capable of packaging Fas and Fas ligand (FasL) into shed vesicles. *In vitro*, shed vesicles containing these proteins are capable of regulating apoptosis (166-168). Fas and FasL are also expressed in chondrocytes and have been implicated in the process of apoptosis in chondrocytes (169, 170). While it is not known whether matrix vesicles contain Fas, FasL or other regulators of apoptosis, apoptosis is highly regulated in the growth plate in a pattern that correlates with the spatially regulated biogenesis of matrix vesicles (84).

Matrix vesicles have mainly been looked at for their role in calcification. Because of the similarities between matrix vesicles and non-skeletal extracellular vesicles, the possibility that vesicles shed by non-skeletal tissues could play a role in pathologic calcification should be further examined. Ultrastructural studies have long shown that matrix vesicles are involved in calcification of arteriosclerotic arteries (171). Interestingly, extracellular membrane vesicles may also play a role in tumor calcification. As previously mentioned, tumor cells shed vesicles at a faster rate than normal cells. Lerner *et al.* showed that alkaline phosphatase is enriched in vesicles shed by human breast cancer cells (172). As discussed above, alkaline phosphatase has been implicated as a key enzyme in matrix vesicle calcification. The presence of alkaline phosphatase in tumor-derived vesicles, suggests that they also have the potential to initiate calcification. There have been several studies that support this theory. Extracellular tumor-derived vesicles have been implicated as initial sites of calcification of psammoma bodies. The formation of psammoma bodies is a common mechanism of tumor calcification. Lipper *et al.* showed that extracellular vesicles, formed from intact human meningioma cells, were associated with psammoma bodies and appeared instrumental in the calcification of extracellular material *in vitro* (173). In an ultrastructural study of mineralizing craniopharyngiomas, needle-shaped crystals similar to hydroxyl-apatite were found in membrane bound vesicles morphologically similar to matrix vesicles (174). Similar findings were seen in oligodendrogliomas (175), and arachnoid villi (176).

With this compelling evidence, it appears that the study of extracellular plasma membrane derived vesicles is

important for understanding the functions of both skeletal and non-skeletal tissues, under normal and pathologic conditions. In the future, there is much to be learned by comparing skeletal and non-skeletal matrix vesicle function and biogenesis.

8. PERSPECTIVE

Matrix vesicle initiation of biomineralization in growth plate cartilage and newly-forming bone and dentin is further documented in this review. However, our understanding of the mechanisms of matrix vesicle biogenesis and mineralization is, at present, incomplete. The balance of evidence indicates that MV biogenesis is polarized and spatially-oriented, suggesting that selected areas of the mother cell surface membrane are preferentially enriched in proteins (phosphatases, etc.) and lipids (lipid rafts) to facilitate MV mineral-initiation after the nascent MV has budded from these selected membrane sites. Important analogies probably exist between general mechanisms controlling membrane biogenesis and exocytosis and the process of MV biogenesis. Thus, molecules are considered that are known to mediate membrane contraction, fusion and vesicle budding in a number of non-skeletal biological systems. Calcifying matrix vesicles are not unique. Other types of functional, extracellular, plasma membrane vesicles exist that arise from non-mineralizing tissues, as discussed above. Lessons learned about the biogenesis or function of these non-skeletal matrix vesicles may apply to skeletal MVs as well. It is interesting that mineral-initiation is not the only important function of MVs. In growth plate, MVs are also carriers of 1) BMPs that probably promote the morphogenesis of the underlying, newly-forming bone of the metaphysis, 2) VEGF, a promoter of metaphyseal angiogenesis, is also likely to be present in MVs, since it is most highly expressed in MV-releasing chondrocytes of the growth plate (although this has not been confirmed experimentally) and 3) matrix metalloproteinases, especially MMPs 2, 9 and 13, which can facilitate terminal chondrocyte differentiation, as well as achieving the orderly digestion of cartilage matrix prior to its replacement by ingrowing new bone. Finally, recent data is presented, which substantiates the importance of MV phosphatases (especially alkaline phosphatase) in promoting and regulating MV mineralization. Hopefully, this review will suggest new perspectives for future researchers, who may be fascinated by the concept of extracellular vesicles, such as MVs, being responsible for important metabolic tasks, and, thus, be motivated to further our understanding of such gifted vesicles.

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