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Laboratory Investigations

Matrix Vesicles Produced by Osteoblast-Like Cells in Culture Become Significantly Enriched in Proteoglycan-Degrading Metalloproteinases after Addition of β-Glycerophosphate and Ascorbic Acid

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Abstract. Matrix vesicles, media vesicles, and plasma membranes from three well-characterized, osteoblast-like cells (ROS 17/2.8, MG-63, and MC-3T3-E1) were evaluated for their content of enzymes capable of processing the extracellular matrix. Matrix vesicles were enriched in alkaline phosphatase specific activity over the plasma membrane and contained fully active neutral, but not acid, metalloproteinases capable of digesting proteoglycans, potential inhibitors of matrix calcification. Matrix vesicle enrichment in neutral metalloproteinase varied with the cell line, whereas collagenase, lysozyme, hyaluronidase, and tissue inhibitor of metalloproteinases (TIMP) were not found in any of the membrane fractions examined. MC-3T3-E1 cells were cultured for 32 days in the presence of ascorbic acid (100 μg/ml), β-glycerophosphate (5 mM), or a combination of the two, to assess changes in matrix vesicle enzymes during calcification. Ascorbate or β-glycerophosphate alone had no effect, but in combination produced significant increases in both active and total neutral metalloproteinase in matrix vesicles and plasma membranes, with the change seen in matrix vesicles being the most dramatic. This correlated with an increase in the formation of von Kossa-positive nodules. The results of the present study indicate that osteoblast-like cells produce matrix vesicles enriched in proteoglycan-degrading metalloproteinases. In addition, the observation that matrix vesicles contain significantly increased metalloproteinases under conditions favorable for mineralization *in vitro* lends support to the hypothesis that matrix vesicles play an important role in extracellular matrix processing and calcification in bone.

Key words: Matrix vesicles — Osteoblast-like cells — Metalloproteinases — Ascorbic acid — β-Glycerophosphate.

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Matrix vesicles are extracellular organelles produced by cells that mineralize their matrix [1–3]. It has been hypothesized that these organelles are either initial sites of calcification *in vivo* or intimately associated with these sites [4, 5]. They have been found to be enriched in alkaline phosphatase [6–11], proteolipids [12], and calcium-phospholipid- phosphate complexes [13, 14]. In addition, hormones such as vitamin D have been found to regulate matrix vesicle-associated enzymes such as alkaline phosphatase and phospholipase A_2 [9–11, 15–19]. In the electron microscope, hydroxyapatite crystals have been observed on the inner leaflet of the matrix vesicle membrane [20].

It is also likely that matrix vesicles are involved in preparing the extracellular matrix for mineralization. It has been recognized that proteoglycan aggregates are inhibitors of mineralization and that they must be removed for mineralization to begin [21–25]. Hirshman et al. [26] found neutral proteases in matrix vesicles and suggested that they may participate in matrix processing. Katsura and Yamada [27] have made similar observations utilizing matrix vesicles from chick growth plates, and Einhorn et al. [28] have postulated that matrix vesicle-derived proteases may also be involved in fracture callus remodeling. Consistent with these observations, we have recently determined that matrix vesicles produced by growth zone chondrocytes in culture are enriched in metalloproteinases capable of digesting proteoglycan [29].

With these observations in mind, we hypothesized that extracellular matrix processing enzymes may also be found in osteoblast-like cell-derived matrix vesicles and that the stage of osteoblast differentiation might also affect the profile of enzymes present and/or their activity [30]. In addition, if matrix processing is critical for mineralization, then factors that increase the amount or rate of mineralization should also increase the amount of these enzyme activities found in the matrix vesicles. To explore these questions, matrix vesicles and plasma membranes from three well-characterized,

osteoblast-like cell lines (ROS 17/2.8, MG-63, and MC-3T3-E1) derived from three different species and stages of osteogenic differentiation were examined for their content of matrix processing enzymes. The addition of ascorbic acid and β-glycerophosphate to the culture medium of MC-3T3-E1 cells was also evaluated for its effect on matrix vesicle and plasma membrane enzyme content, as both of these agents are believed to be involved in matrix protein synthesis and/or calcification in osteogenic cells.

Materials and Methods

Culture of Osteoblast-Like Cells

Three previously characterized osteoblast-like cell lines were used in this study. ROS 17/2.8 cells were originally isolated from a rat osteosarcoma and are characterized by displaying receptors for $1,25(OH)_2D_3$ [31] and glucocorticoids [32], as well as increased amounts of bone alkaline phosphatase activity in response to $1,25(OH)_2D_3$ [33]. The second osteoblast-like cell line is an osteogenic, nontransformed murine cell, MC-3T3-E1, cloned from newborn mouse calvaria by Kodama et al. [34]. These cells have low levels of alkaline phosphatase when they are subconfluent, but this changes rapidly to increased levels at confluence. The cell line has retained other osteoblast characteristics such as receptors for $1,25(OH)_2D_3$ and parathyroid hormone, ability to synthesize type I collagen, production of bone type alkaline phosphatase, and the ability to calcify in culture [35–37]. The third osteoblast-like cell line is a relatively new isolate called MG-63 which was isolated from a human osteosarcoma [38]. Upon treatment with $1,25(OH)_2D_3$, it produces increased amounts of alkaline phosphatase, and cell proliferation is inhibited [16, 17, 38]. We have previously reported that all three cell lines produce matrix vesicles that respond to $1,25(OH)_2D_3$ by producing increased alkaline phosphatase mRNA, protein [16] and/or specific activity [17, 18]. All culture conditions outlined below were optimized for production of alkaline phosphatase by each cell line.

ROS 17/2.8 cells (a gift from Dr. Gideon Rodan at Merck, Sharpe and Dohme, West Point, PA) were plated in T-150 flasks at an initial cell density of 20,000 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) plus 1% streptomycin-fungizone-gentamycin and cultured for 24 hours at 37°C in 5% CO_2 and 100% humidity to allow the cells to attach. Fresh medium containing 10% FBS was then added, incubation continued for an additional 48 hours, and the cells were harvested as described below.

MG-63 cells were plated in T-150 flasks at an initial density of 9300 cells/cm^2 in DMEM containing 10% FBS and antibiotics and cultured for 2 days at 37°C in 5% CO_2. Fresh medium containing 10% FBS was then added, incubation continued for an additional 4 days, and the cells were harvested as described below. The initial culture of MG-63 cells was obtained from the American Type Culture Collection (Rockville, MD).

MC-3T3-E1 cells (a gift from Dr. H. Kodama, Ohu University, Fukishima, Japan) were plated in T-150 flasks at an initial density of 20,000 cells/cm^2 in DMEM containing 10% FBS and antibiotics and cultured for 2 days at 37°C in 5% CO_2. Fresh medium containing 10% FBS was then added, incubation was continued for an additional 10 days, and the cells were harvested as described below.

As a model for mineralization, MC-3T3-E1 cells were plated as before. The medium was then removed and four different types of fresh medium was added containing (1) α-MEM (Gibco) containing 10% FBS (= control medium); (2) control medium plus 100 μg/ml ascorbic acid; (3) control medium plus 5 mM β-glycerophosphate; (4) control medium plus 100 μg/ml ascorbic acid and 5 mM β-glycerophosphate. Fresh medium was added two times per week and the cells were harvested at 32 days when they had begun to form von Kossa-positive nodules. The concentration of ascorbic acid and β-glycerophosphate were chosen based on the work of others (see Discussion) and our experience with this cell line and induction of calcification. In addition, we decided upon this experimental design so that we could separate the effect of each addition in relation to the control and combination treatment.

Nodules were assessed with respect to number, total area, and mean size using an automated imaging system consisting of a Jandel Video Analysis (JAVA) program (Jandel Scientific, San Rafael, CA) of images produced using a videoscreen (Sony Trinitron), camera (Sony CCD/RGB), and Olympus BH2 microscope equipped with metallurgical lenses.

Preparation of Media Vesicle, Plasma Membrane, and Matrix Vesicle Fractions

At the end of incubation, the conditioned media were removed and centrifuged at 500 × *g* for 5 minutes to pellet cells. The supernatant was recentrifuged at 21,000 × *g* for 10 minutes to pellet cell debris, including any mitochondria and endoplasmic reticulum. The resulting supernatant was centrifuged at 100,000 × *g* for 1 hour to pellet media vesicles which were then resuspended in 1 ml 0.9% NaCl. Following assay for protein content [39] and alkaline phosphatase specific activity [40], the remaining membranes were stored at −70°C.

The cell layer, including extracellular matrix and cells, was washed twice with serum-free DMEM and then trypsinized (0.25% in Hank's balanced salt solution, HBSS). Following addition of FBS to inhibit trypsin, cells were separated from the trypsin digest by centrifugation for 10 minutes at 500 × *g*; pelleted cells were resuspended in HBSS and counted. Plasma membranes were then prepared from this resuspended cell pellet by the method of Fitzpatrick et al. [41]. Following resuspension of the plasma membranes in 1 ml 0.9% NaCl, protein content [39] and alkaline phosphatase specific activity [40] were determined. The remaining membrane fractions were stored at −70°C.

The supernatant of the 500 × *g* centrifugation of the trypsinized cell layer was centrifuged again at 21,000 × *g* for 10 minutes to pellet cell debris, including any mitochondria and endoplasmic reticulum. The resulting supernatant was recentrifuged at 100,000 × *g* for 1 hour to pellet matrix vesicles [42]. After resuspending the matrix vesicles in 1 ml 0.9% NaCl, protein content and alkaline phosphatase were assayed as described above and the remaining matrix vesicles were stored at −70°C.

Previous studies have shown that matrix vesicles produced by osteoblast-like cells in culture [17] exhibit a morphology typical of classic matrix vesicles isolated from cartilage and chondrocyte cultures. Nonetheless, to assure that reliable membrane preparations were obtained, only matrix vesicle preparations having an alkaline phosphatase specific activity greater than twofold higher than their respective plasma membranes and free of INT-reductase activity, a marker for mitochondrial contamination, were used for these studies.

Extraction of Enzymes and Tissue Inhibitor of Metalloproteinases (TIMP)

The choice of conditions under which the enzymes and TIMP were to be extracted was determined by using the procedure previously utilized for optimizing extraction of these same macromolecules from chondrocyte-derived media vesicles, plasma membranes, and matrix vesicles [29]. Five different extraction buffers were tested on the osteoblast-like cell-derived membrane fractions. In all cases, the extraction buffers and method, previously found to be optimal for chondrocyte membrane fractions, also performed well with the osteoblast-like cell membranes.

The final method consists of dividing 1-ml samples of matrix vesicles or plasma membranes (≈1 T-150 flask) into three aliquots. The first aliquot (100 μl) was left in 0.9% NaCl and assayed for protein [39] and alkaline phosphatase [40], as described above. A second aliquot (200 μl) was mixed with an equal volume of 0.2 M sodium acetate buffer, pH 4.5, containing 0.6 M NaCl and 0.4% Triton X-100. The membrane suspension was briefly mixed for 20–30 seconds with a ground glass homogenizer (Duall #20, Kontes Co., Vineland, NJ) and then gently stirred for 2 hours at 4°C. The

extract was then centrifuged at 106,000 × g for 1 hour and the supernatant was dialyzed into β-D-glucuronidase assay buffer (see below). The third aliquot (700 μl) was extracted in a similar fashion, but using 0.1 M Tris buffer, pH 7.5, containing 4 M guanidine HCl, 0.02 M $CaCl_2$, and 0.4% Triton X-100. Supernatants were dialyzed into metalloproteinase or plasminogen activator assay buffer (see below).

Specific Biochemical Analyses

Neutral and Acid Metalloproteinase. Neutral and acid metalloproteinase activities were assayed on proteoglycan-containing beads as described by Dean et al. [43]. The assays are based on the digestion of ^{3}H-proteoglycan monomer which is entrapped in polyacrylamide beads. The pore size of the beads is adjusted so that the undigested monomers are retained inside the beads, whereas digestion fragments of 200,000 daltons or less are allowed to diffuse out. The assay measures the digestion of proteoglycan core protein and can detect approximately 0.3 ng trypsin in 2 hours at 37°C.

Samples were dialyzed into 0.05 M Tris, pH 7.5, buffer containing 0.01 M $CaCl_2$, 0.2 M NaCl, and 0.05% Brij 35, for assay of neutral metalloproteinase or into 0.1 M Tris-maleate buffer, pH 5.5, containing 0.01 M $CaCl_2$ and 0.05% Brij 35, for assay of acid metalloproteinase. Each sample (10–30 μl) was added to a 7-ml scintillation vial containing 2 mg ^{3}H-proteoglycan beads (12,000 cpm/mg; 150 μg proteoglycan/mg beads) and made up to a total volume of 200 μl. Active enzyme was measured without any other addition to the vial, whereas total enzyme (latent + active forms) was measured in the presence of 1.0 mM aminophenylmercuric acetate (APMA). Blanks contained 1.0 mM 1,10-phenanthroline, a zinc chelator, to inhibit all metalloproteinase activity. All acid metalloproteinase vials contained 1 μg pepstatin to inhibit cathepsin D. Incubation was continued for 18 hours at 37°C. At the end of incubation, 6.0 ml Aquasol (New England Nuclear, Boston, MA) scintillation fluid was added and the vials were counted. Enzyme activity was expressed as U/mg protein, where one enzyme unit released 1 μg ^{3}H-proteoglycan/minute at 37°C from 2-mg beads.

Collagenase. Collagenase was assayed on telopeptide-free ^{3}H-acetylated type I collagen by the method of Dean and Woessner [44], as modified in Dean et al. [45]. The method is sensitive and specific for collagenase and only detects hydrolysis of the helix.

Tissue Inhibitor of Metalloproteinases. TIMP was measured using active collagenase extracted from the growth plate of rachitic rats [45] because the high level of neutral metalloproteinase found in matrix vesicles and plasma membrane samples interfered with the Azocoll assay used in earlier studies [46]. Collagenase was activated by treatment with 0.5 mM APMA for 1 hour at 37°C and then exhaustively dialyzed against neutral metalloproteinase assay buffer.

For assay, active collagenase [9 enzyme units (1 enzyme unit equals 1 ng collagen digested/minute at 30°C)], inhibitor (0–20 μl), and neutral metalloproteinase assay buffer to a final volume of 50–60 μl were mixed together and preincubated for 1 hour at 37°C to allow enzyme and inhibitor to react. After preincubation, 32 μg ^{3}H-collagen (see above) and buffer sufficient to make a final volume of 110 μl were added, and the incubation was continued at 30°C for 22 hours. Enzyme blanks contained 1 mM 1,10-phenanthroline. At the end of incubation, the standard collagenase assay was performed as described above. One unit of inhibitor blocked one unit of enzyme.

Plasminogen Activator. Assay of the serine proteinase, plasminogen activator, was conducted according to the method of Coleman and Green [47], with urokinase (#672081, Calbiochem, LaJolla, CA) as a standard. The assay consists of two discrete steps. In the first step, plasminogen is converted to plasmin by any plasminogen activators present in the sample. Plasmin is then measured by its ability to hydrolyze thiobenzylbenzyloxycarbonyl-L-lysinate (Z-Lys-SBzl), which reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to form a chromogen that can be read spectrophotometrically at 412 nm. The second step is maximized for plasmin activity and minimized for plasminogen activator activity.

Table 1. Enzyme content of media vesicles and matrix vesicles isolated from cultures of MG-63 osteoblast-like cells[a]

		Matrix vesicles	Media vesicles
Alkaline phosphatase (μmol P_i/mg protein/min)		17.8 ± 2.8[b]	3.5 ± 0.5
Neutral metalloproteinase (enzyme U/mg protein)	Active	7.4 ± 3.1[b]	1.5 ± 0.4
	Total	3.8 ± 1.1[b]	1.4 ± 0.4
Acid metalloproteinase (enzyme U/mg protein)	Active	2.4 ± 1.2	2.5 ± 1.1
	Total	4.0 ± 2.3	4.0 ± 1.6
Plasminogen activator (enzyme U/mg protein)		0.8 ± 0.3[b]	2.0 ± 0.8

[a] All values are the mean ± SEM for n = 6 samples where each sample represents the membranes isolated from a single culture (T-150 flask). Data are from one of two replicate experiments. A similar distribution of enzymes between matrix vesicles and media vesicles was also found in cultures of ROS 17/2.8 and MC-3T3-E1 cells. The only exception to this generalization was found in MC-3T3-E1 cells where no enrichment in plasminogen activator was observed in the media vesicles

[b] Differences between media vesicles and matrix vesicles were significant at $P < 0.05$

β-D-Glucuronidase. Assay of β-D-glucuronidase was performed by a modification of the method of Himeno et al. [48]. Extracts were dialyzed against 0.2 M sodium acetate buffer, pH 4.5, overnight at 4°C. The extract (40 μl) was added to 210 μl 0.2 M sodium acetate buffer, pH 4.5, containing 0.1% bovine serum albumin and 10 mM p-nitrophenyl β-D-glucosiduronic acid (Sigma, St. Louis, MO) and incubated for 2 hours at 37°C. The reaction was terminated by the addition of 0.9 ml 0.1 M NaOH, and the p-nitrophenol generated was quantitated spectrophotometrically at 400 nm. One unit of activity generated 1 nmol p-nitrophenol/hour. β-glucuronidase (Type B-1; Sigma) was used as a standard.

Hyaluronidase. Hyaluronidase activity was measured by a simple electronic capillary microviscometer, as described by Muller and Pita [49]. Human umbilical cord hyaluronic acid (200 μg; Sigma) was mixed with 0.1 M sodium acetate buffer, pH 4.5, containing 0.15 M NaCl and sample in a final volume of 0.2 ml and incubated for 22 hours at 37°C.

Lysozyme. Lysozyme was measured according to the method of Sorgente et al. [50] using *Micrococcus lysodeikticus* agar diffusion plates with egg white lysozyme (Sigma) as a standard. Samples were tested directly in the presence of 2 M guanidine buffer. Plates were incubated for as long as 41 hours at 37°C before measuring zones of clearing. The assay was capable of detecting 3.6 μg/ml lysozyme.

Statistical Analyses

For media vesicle, matrix vesicle, or plasma membrane enzyme activity, each data point represents the mean ± SEM for six samples, where each sample is equivalent to the amount of membrane(s) obtained from one T-150 flask. Statistical significance was determined by comparing each data point to the control using the Bonferroni t-test. Differences were considered significant at $P < 0.05$.

Results

Enzyme Content of Membrane Fractions Produced by ROS17/2.8, MG-63, and MC-3T3-E1 Osteoblast-Like Cells in Culture

Comparison of Media Vesicle and Matrix Vesicle Enzyme Content. Both media vesicles and matrix vesicles were iso-

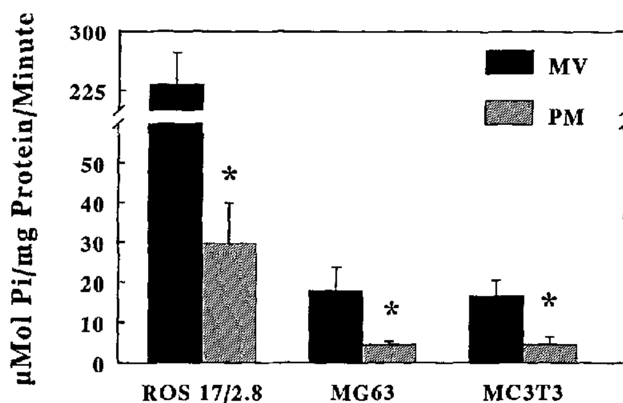

Fig. 1. Alkaline phosphatase specific activity of matrix vesicles (MV) and plasma membranes (PM) isolated from confluent cultures of ROS 17/2.8, MG-63, and MC-3T3-E1 cells. Data represent the mean ± SEM for n = 6 samples where each sample is from a single culture. Data are from one of two replicate experiments. $*P < 0.05$ for MV versus PM.

lated from confluent cultures of all three osteoblast-like cell lines. Enzyme content for each type of vesicle produced by MG-63 osteoblast-like cells is shown in Table 1. It was found that alkaline phosphatase specific activity was fivefold higher in matrix vesicles than in media vesicles. Active and total neutral metalloproteinase specific activity was also significantly enriched in matrix vesicles compared with media vesicles. Of particular importance was the finding that all neutral metalloproteinase in the matrix vesicles was fully active. Total enzyme appeared to be present at lower levels than active enzyme in MG-63 osteoblast-like cells and may be the result of APMA-induced destruction of fully active enzyme during the assay, but this was not statistically significant. In contrast to the neutral enzyme, both forms of acid metalloproteinase were found in equal amounts in both types of vesicles, whereas media vesicles were enriched 2.6-fold in plasminogen activator content.

A similar distribution of enzyme specific activity into media vesicles and matrix vesicles was observed for membranes isolated from ROS 17/2.8 and MC-3T3-E1 osteoblast-like cell cultures. The only inconsistency observed was that plasminogen activator levels were similar in both media vesicles and matrix vesicles produced by MC-3T3-E1 cells.

Enzyme Content of Matrix Vesicles and Plasma Membranes Produced by ROS 17/2.8, MG-63, and MC-3T3-E1 Osteoblast-Like Cells in Culture. All three cell lines produced matrix vesicles enriched in alkaline phosphatase specific activity (Fig. 1). Matrix vesicles isolated from cultures of ROS 17/2.8 cells displayed eightfold enrichment in alkaline phosphatase specific activity over the plasma membrane. Matrix vesicles produced by MG-63 and MC-3T3-E1 cells also contained increased amounts of alkaline phosphatase, but their specific activity was four times that found in the plasma membrane. Interestingly, the plasma membranes from each type of osteoblast-like cell contained different amounts of alkaline phosphatase specific activity, with those isolated from ROS 17/2.8 cells containing seven times the amount found in either MG-63 or MC-3T3-E1 cells. These results were similar to those previously reported by us [17] and demonstrate that the membrane preparations used for metalloproteinase assays were typical of matrix vesicles and plasma membranes used in other studies.

Active and total neutral metalloproteinase activity was found at higher specific activity in matrix vesicles than

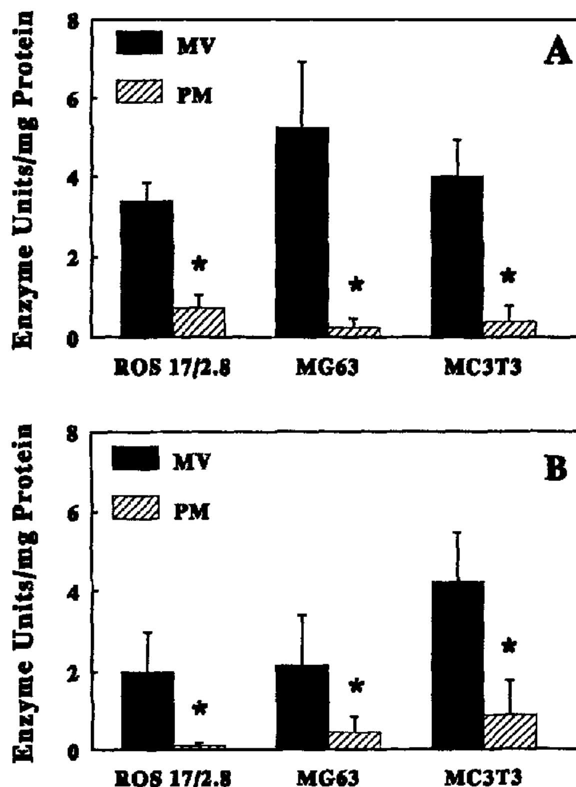

Fig. 2. Neutral metalloproteinase activity extracted from osteoblast-like cell-derived matrix vesicles (MV) and plasma membranes (PM) and assayed using proteoglycan-containing polyacrylamide gel beads. (**A**) Content of active enzyme found in each membrane fraction for ROS 17/2.8, MG-63, and MC-3T3-E1 cells. (**B**) Content of total (active + latent) enzyme found in each membrane fraction for ROS 17/2.8, MG-63, and MC-3T3-E1 cells. In both panels, osteoblast-like cells were grown to confluence and membranes were isolated as described in Materials and Methods. Activity is shown as enzyme U/mg protein. All data represent the mean ± SEM for n = 6 samples where each sample is from a single culture. Data are from one of two replicate experiments. $*P < 0.05$ for MV versus PM.

plasma membranes (Fig. 2). Matrix vesicles from ROS 17/2.8 cells contained 4.6-fold more active (Fig. 2A) and 21-fold more total (Fig. 2B) neutral metalloproteinase specific activity than their respective plasma membranes. Active enzyme was also enriched in matrix vesicles from MG-63 (21-fold) and MC-3T3-E1 (10-fold) cells, whereas total enzyme for both of these cell lines was only enriched 5-fold. All cells produced matrix vesicles containing fully active neutral metalloproteinase, but the level of active enzyme was widely variable and dependent on cell type. Similarly, total enzyme levels varied and, in the case of MG-63 and ROS 17/2.8, osteoblast-like cells were lower than found for the active form, probably due to APMA-induced destruction during the assay (see above). However, no statistically significant differences between total and active enzyme activity were noted.

Matrix vesicles and plasma membranes from the three cell lines were also assayed for their content of active and total acid metalloproteinase (Fig. 3). As before, matrix vesicles contained metalloproteinase that was fully active, but no enrichment of enzyme specific activity was observed. The plasminogen activator content of osteoblast-like, cell-derived matrix vesicles and plasma membranes was also measured (Fig. 4). No membrane-specific enrichment was observed for any of the cell types examined.

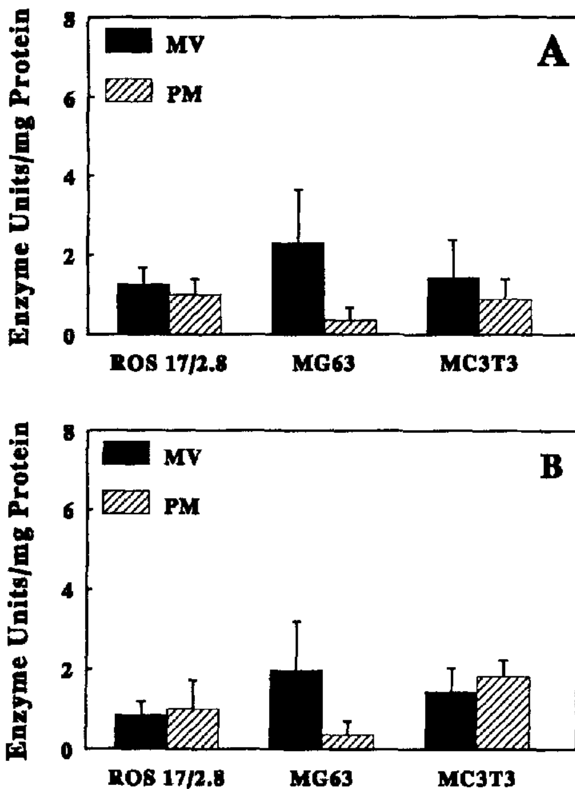

Fig. 3. Acid metalloproteinase activity extracted from osteoblast-like cell-derived matrix vesicles (MV) and plasma membranes (PM) and assayed using proteoglycan-containing polyacrylamide gel beads. (A) Content of active enzyme found in each membrane fraction for ROS 17/2.8, MG-63, and MC-3T3-E1 cells. (B) Content of total (active + latent) enzyme found in each membrane fraction for ROS 17/2.8, MG-63, and MC-3T3-E1 cells. In both panels, osteoblast-like cells were grown to confluence and membranes were isolated as described in Materials and Methods. Activity is shown as enzyme U/mg protein. All data represent the mean ± SEM for n = 6 samples where each sample is from a single culture. Data are from one of two replicate experiments. $*P < 0.05$ for MV versus PM.

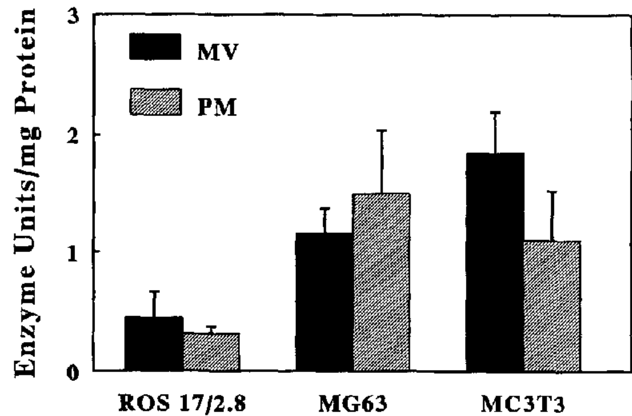

Fig. 4. Plasminogen activator activity extracted from osteoblast-like cell-derived matrix vesicles (MV) and plasma membranes (PM). Cells were grown to confluence and membranes were isolated as described in Materials and Methods. Activity is shown as enzyme U/mg protein. All data represent the mean ± SEM for n = 6 samples where each sample is from a single culture. Data are from one of two replicate experiments. No significant differences were observed between PM and MV for each cell line.

Effect of Ascorbic Acid and β-Glycerophosphate on Calcification in Long-Term Cultures of MC-3T3-E1 Cells

Effect of Ascorbic Acid and β-Glycerophosphate on von Kossa-Positive Nodule Formation. Formation of von Kossa-positive nodules in long-term cultures of MC-3T3-E1 cells was dependent on the presence of both ascorbic acid and β-glycerophosphate in the medium (Fig. 5). When MC-3T3-E1 cells were cultured for 32 days in either DMEM containing 10% FBS or DMEM with 10% FBS and 100 μg/ml ascorbic acid, essentially no Von Kossa-positive nodules were observed. Addition of β-glycerophosphate to the serum-containing medium resulted in nodule formation as demonstrated by an increase in nodule number (Fig. 5A) and total area occupied by the nodules (Fig. 5B), but this increase was not statistically different from the control cultures. When the cells were cultured in medium containing both β-glycerophosphate and ascorbic acid, there was a significant increase in both nodule number and total area of nodules formed. Though mean nodule size was comparable in cultures with β-glycerophosphate alone or with β-glycerophosphate and ascorbate, the variation in size was more pronounced in those cells treated with β-glycerophosphate alone (Fig. 5C).

Effect of Ascorbic Acid and β-Glycerophosphate on Enzyme Content of Matrix Vesicles and Plasma Membranes Isolated From MC-3T3-E1 Cell Cultures. Incubation of MC-3T3-E1 cells with either ascorbic acid, β-glycerophosphate, or a combination of the two increased cell number twofold (Table 2). As variation in protein content between the treatment groups was greater than twofold, enzyme activity was presented as specific activity.

As described before, alkaline phosphatase specific activity was enriched in matrix vesicles over that found in the plasma membranes (Fig. 6). Ascorbic acid, β-glycerophosphate, or a combination of the two, however, did not significantly alter the specific activity of the enzyme found in either matrix vesicles or plasma membranes.

Active and total neutral metalloproteinase specific activity was found at significantly higher levels in matrix vesicles than plasma membranes (Fig. 7). Irrespective of the culture conditions, matrix vesicles were always enriched in fully activated neutral metalloproteinase. The plasma membrane fraction, however, displayed some variation in the proportion of active enzyme. Compared with untreated controls, the addition of ascorbic acid to the culture medium significantly reduced active and total neutral metalloproteinase specific activity in matrix vesicles, but not plasma membranes. Addition of β-glycerophosphate, by itself, to the medium had no effect on metalloproteinase specific activity in either membrane fraction compared with control. When added together with ascorbic acid, however, significant increases in both active and total metalloproteinase were observed for both membrane fractions. Matrix vesicles contained three times more active and total enzyme than matrix vesicles produced by the untreated cells or those treated only with β-glycerophosphate. Enzyme activity of the plasma membrane was increased six- to eightfold, but the specific activity was only 25 to 50% of that found in the matrix vesicles.

Active and total acid metalloproteinase specific activities were not enriched in matrix vesicles compared with plasma membranes (Fig. 8). The only exception to this generalization was the β-glycerophosphate-treated cultures, where active enzyme was found at significantly higher specific activ-

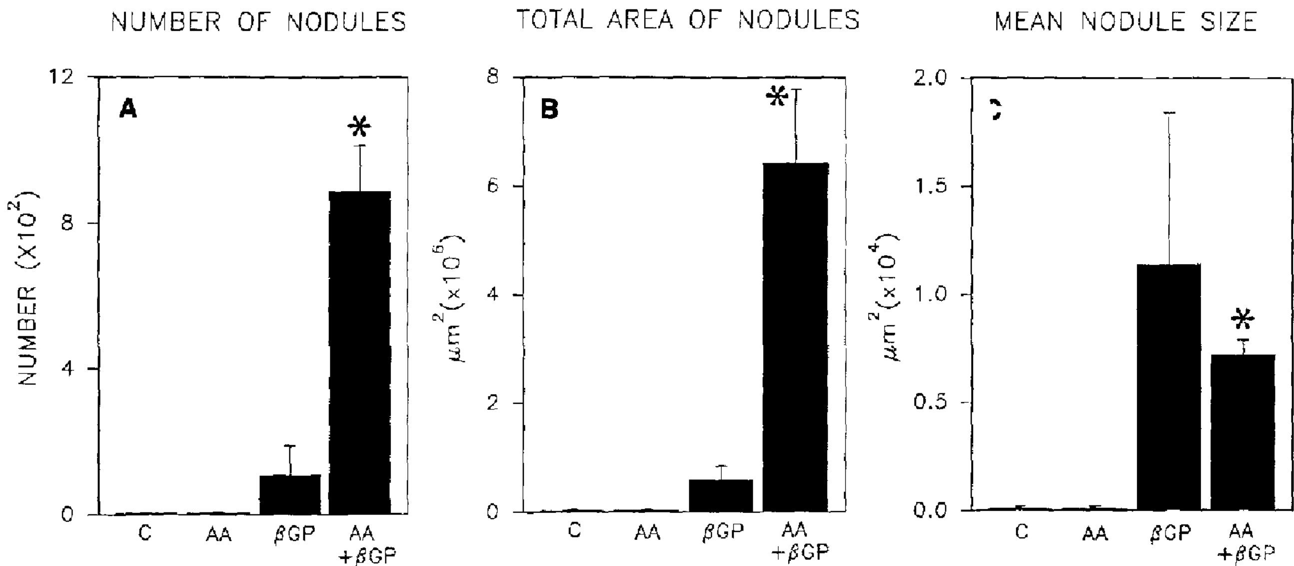

Fig. 5. Effect of ascorbic acid (AA) and β-glycerophosphate (βGP) on von Kossa-positive nodule formation in MC-3T3-E1 cells after 32 days in culture. Cells were cultured as described in Materials and Methods. At the end of culture, the cells were fixed *in situ* using buffered formalin and then stained with von Kossa. Using an automated imaging system, nodule number (A), total nodule area (B), and mean nodule size (C) were determined. All data represent the mean ± SEM for n = 3 cultures. *$P < 0.05$ for treatment versus control.

Table 2. Effect of ascorbic acid and β-glycerophosphate on cell number in cultures of MC-3T3-E1 osteoblast-like cells[a]

	Cell number/ T-150 Flask (×10^6)
Untreated	12.9 ± 1.9
Treated	
+ Ascorbic acid (100 μg/ml)	25.1 ± 1.1[b]
+ β-glycerophosphate (5 mM)	22.7 ± 1.4[b]
+ Ascorbic acid & β-glycerophosphate (100 μg/ml + 5 mM)	22.1 ± 1.4[b]

[a] MC-3T3-E1 cells were cultured in the presence or absence of ascorbic acid, β-glycerophosphate, or a combination of the two for 32 days, trypsinized, and counted. All values are the mean ± SEM for n = 6 cultures and are from one of two replicate experiments.
[b] Compared with untreated cultures, differences were significant at $P < 0.05$

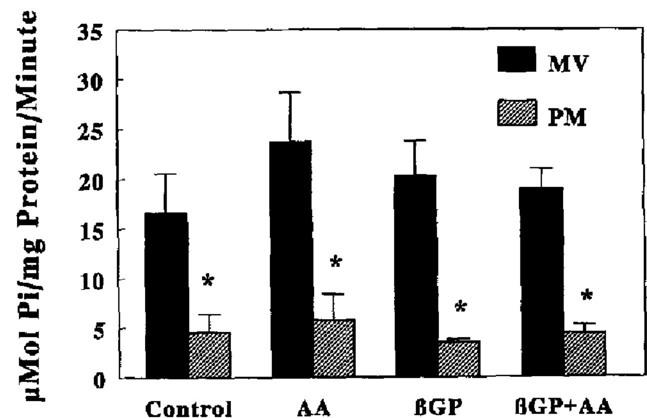

Fig. 6. Effect of ascorbic acid (AA) and β-glycerophosphate (βGP) on alkaline phosphatase specific activity of matrix vesicles (MV) and plasma membranes (PM) isolated from MC-3T3-E1 cells after 32 days in culture. Cells were grown and membranes were isolated as described in Materials and Methods. Data represent the mean ± SEM for n = 6 samples where each sample is from a single culture. Data are from one of two replicate experiments. *$P < 0.05$ for MV versus PM.

ity in matrix vesicles than plasma membranes. No other changes achieved statistical significance, but two trends were observed: a clear reduction of both active and total enzyme in the membrane fractions was seen with ascorbic acid treatment, whereas β-glycerophosphate appeared to increase the metalloproteinase specific activity of matrix vesicles.

Plasminogen activator levels were generally unaffected by changes in culture conditions (Fig. 9). However, ascorbic acid treatment significantly reduced matrix vesicle plasminogen activator specific activity compared with the plasma membrane.

Matrix Processing Enzymes Not Found in Osteoblast-Like Cell-Derived Matrix Vesicles or Plasma Membranes. Other enzymes that may have a role in preparing the matrix for calcification were also examined. Collagenase, lysozyme, and hyaluronidase were not detected in this study. TIMP was not found in either matrix vesicles or plasma membranes of any osteoblast-like cells examined. β-D-glucuronidase was used to obtain an index of lysosomal contamination. No activity was detected in any membrane fraction tested.

Discussion

Although matrix vesicles have been associated with mineralization of the extracellular matrix [1, 2], their role is unclear. *In vivo* studies of bone healing have shown that increases in matrix vesicle enzymes, like alkaline phosphatase and phospholipase A_2, correlate well with the formation of

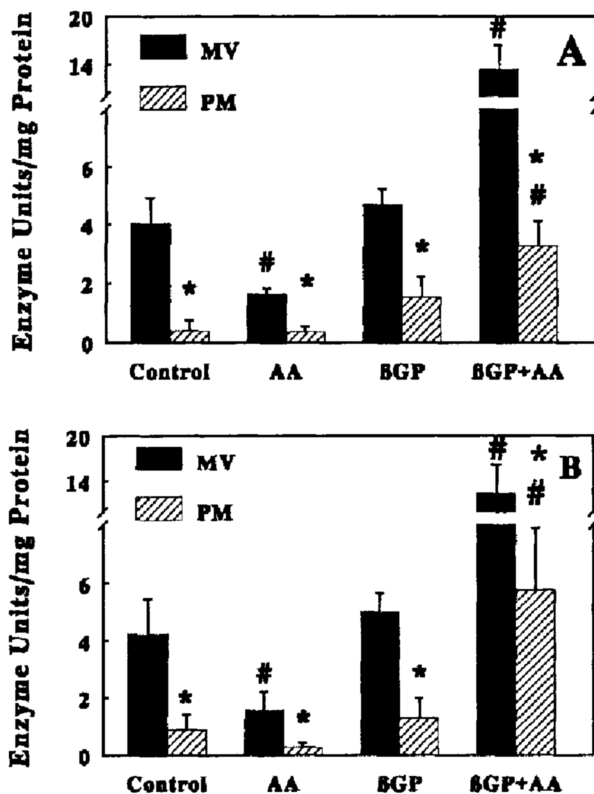

Fig. 7. Neutral metalloproteinase activity extracted from matrix vesicles (MV) and plasma membranes (PM) produced by MC-3T3-E1 cells treated with ascorbic acid (AA) and β-glycerophosphate (βGP) for 32 days in culture. MV and PM were isolated and then extracted and assayed for neutral metalloproteinase activity on proteoglycan-containing polyacrylamide beads as described in Materials and Methods. (**A**) Content of active enzyme found in each membrane fraction. (**B**) Content of total (active + latent) enzyme found in each membrane fraction. In both panels, activity is shown as enzyme U/mg protein. All data represent the mean ± SEM for n = 6 samples where each sample is from a single culture. Data are from one of two replicate experiments. *$P < 0.05$ for MV versus PM; #$P < 0.05$ versus Control.

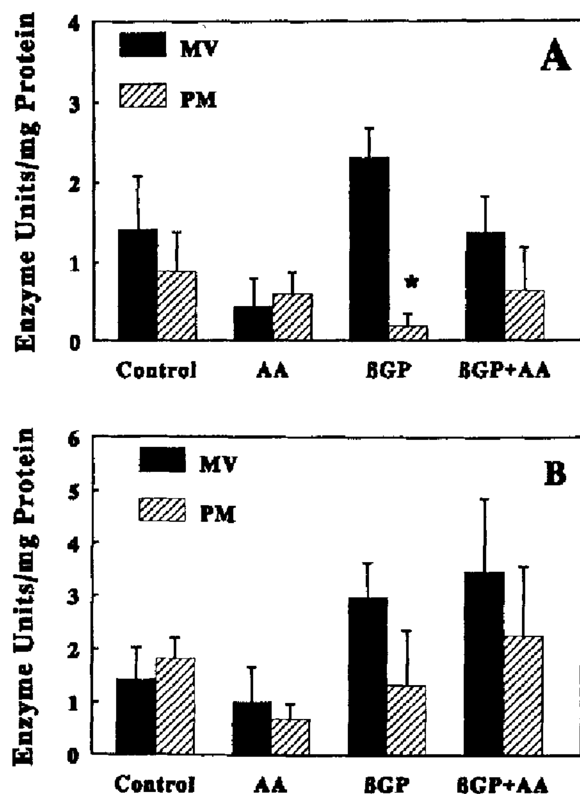

Fig. 8. Acid metalloproteinase activity extracted from matrix vesicles (MV) and plasma membranes (PM) produced by MC-3T3-E1 cells treated with ascorbic acid (AA) and β-glycerophosphate (βGP) for 32 days in culture. MV and PM were isolated and then extracted and assayed for acid metalloproteinase activity on proteoglycan-containing polyacrylamide beads as described in Materials and Methods. (**A**) Content of active enzyme found in each membrane fraction. (**B**) Content of total (active + latent) enzyme found in each membrane fraction. In both panels, activity is shown as enzyme U/mg protein. All data represent the mean ± SEM for n = 6 samples where each sample is from a single culture. Data are from one of two replicate experiments. *$P < 0.05$ for MV versus PM.

hydroxyapatite crystals within the matrix vesicle [51–53]. Similarly, osteoblast-like cell cultures produce matrix vesicles that are enriched in alkaline phosphatase specific activity [17, 18], regulated by hormones and growth factors known to modulate bone formation *in vivo* [16–19], and associated with initial crystal deposition [54]. The present study further strengthens the relationship between matrix vesicles and mineralization of bone by showing that these extracellular organelles contain enzymes capable of processing the extracellular matrix and that the amount of these enzymes is dramatically increased by culture conditions that favor mineralization.

The results demonstrate that osteoblast-like cells produce at least two extracellular membrane-bound vesicles of differing type and composition. The first type is called media vesicles because they are released into the media during culture. They are believed to form when parts of the plasma membrane are sloughed off during culture [42], but their function is unknown. Media vesicles were enriched in plasminogen activator specific activity in two of the three osteoblast cell lines studied. Majeska et al. [55] have similarly reported release of "particulate" alkaline phosphatase activity from ROS 17/2.8 cells stimulated by parathyroid hormone, suggesting that production and release of "membrane" vesicles is not a passive effect of culture, but is under direct cellular control.

The second type of extracellular membrane vesicle, matrix vesicles, is produced by osteoblasts in culture and must be released from the matrix for isolation. Matrix vesicles appear intimately involved in calcification and capable of processing the extracellular matrix, based on their enzyme content. A potential role for these vesicles in calcification is suggested by their metalloproteinase content as specific degradation of noncollagenous proteins such as proteoglycans is required for bulk phase mineral deposition [24, 25, 29, 56, 57]. The fact that neutral metalloproteinase activity in the matrix vesicle is completely masked until the membrane is disrupted and that after the membrane is disrupted the enzyme is released in fully active form suggests that one role for the organelle may be to convey enzyme to the extracellular matrix. According to this hypothesis, control of crystal formation would depend on breakdown of the matrix vesicle membrane, potentially via metabolism of membrane phospholipids [10, 58–60]. Thus, initial crystal formation may be more important for regulating the time of vesicle breakdown and release of active matrix processing proteinases than for nucleating bulk phase calcification.

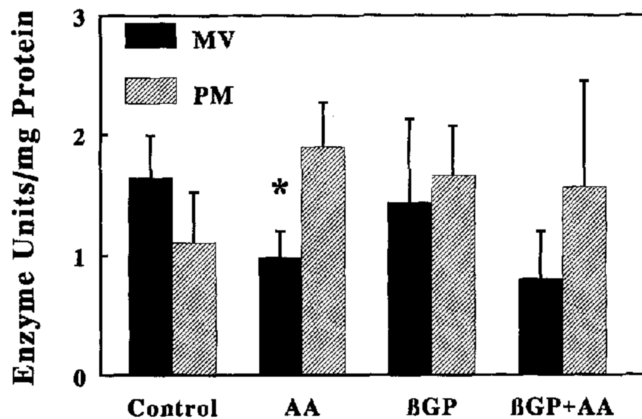

Fig. 9. Plasminogen activator activity extracted from matrix vesicles (MV) and plasma membranes (PM) produced by MC-3T3-E1 cells treated with ascorbic acid (AA) and β-glycerophosphate (βGP) for 32 days in culture. Cells were grown and membranes were isolated as described in Materials and Methods. Activity is shown as enzyme U/mg protein. All data represent the mean ± SEM for n = 6 samples where each sample is from a single culture. Data are from one of two replicate experiments. $*P < 0.05$ for MV versus PM.

Prior studies have demonstrated that matrix vesicle structure and function can be determined by the cells producing them. As a result, it is likely that matrix vesicles can be "primed" or "programmed" to respond in a particular way in the extracellular matrix based on factors incorporated into them at the time of biosynthesis. Changes in membrane composition or enzyme content could be accomplished during biogenesis; in addition, direct regulation of matrix vesicle activity after release into the matrix could also occur. There is ample evidence to suggest that all of these mechanisms may operate *in vivo*. Previous studies have shown that alkaline phosphatase in MG-63 cells (mRNA, protein, and activity) and isolated plasma membranes and matrix vesicles (protein and activity) is regulated by $1,25(OH)_2D_3$ and TGF-β [16]. Furthermore, incubation of isolated osteoblast-derived matrix vesicles with $1,25(OH)_2D_3$ *in vitro* results in a dose-dependent stimulation of alkaline phosphatase specific activity [18]. Taken together, these results suggest that osteoblast-like cells may use matrix vesicles to regulate events at sites distant from the cell.

In the present study, matrix vesicle alkaline phosphatase specific activity was unaffected by addition of ascorbic acid, β-glycerophosphate, or a combination of the two to the medium of MC-3T3-E1 cells. This corroborates the results of Lee et al. [61] who showed that β-glycerophosphate had no effect on a number of parameters associated with calcification, but is in contrast to the observations of Boskey et al. [62], who have shown that cultures incubated with β-glycerophosphate form larger crystals than normally found *in vitro*. We found significant increases in active and total neutral metalloproteinase in matrix vesicles isolated from cultures treated with both ascorbic acid and β-glycerophosphate whereas in untreated cultures or those treated with ascorbic acid or β-glycerophosphate alone, isolated matrix vesicles only contained one-third the amount of active and total enzyme. We interpret these results to indicate that ascorbic acid is necessary for matrix synthesis. β-glycerophosphate increases calcification of the matrix once it is formed, partly by serving as a phosphate source and partly by stimulating matrix degradation, facilitating crystal formation and growth. This hypothesis is further strengthened by our morphologic-observations demonstrating a synergistic increase in von Kossa-positive nodule formation in cultures treated with both ascorbic acid and β-glycerophosphate. A study by Franceschi and Iyer [63], using MC-3T3-E1 cells, demonstrated that ascorbic acid was essential for proper matrix synthesis (i.e., type I collagen). In our studies, no effect on either von Kossa-positive nodule formation or matrix vesicle or plasma membrane enzymes was observed with ascorbate alone. The addition of β-glycerophosphate alone in Franceschi and Iyer's study, as well as our own, was without effect. The combination of ascorbic acid and β-glycerophosphate, however, resulted in an augmentation of mineralization in their study and dramatic increases in the formation of von Kossa-positive nodules and hydroxyapatite and matrix vesicle neutral metalloproteinase content in ours. Taken together, these results suggest that matrix vesicle maturation and subsequent maturation of the matrix can be regulated extracellularly when matrix and ionic (calcium and phosphate) conditions are conducive to calcification.

MC-3T3-E1 cells have also been used to examine other aspects of mineralization including the effects of hormones, such as $1,25(OH)_2D_3$. Matsumoto et al. [64] have shown that MC-3T3-E1 cells begin to deposit large amounts of calcium into both cell and matrix layers after 2 weeks in culture and that $1,25(OH)_2D_3$ stimulates this process in a dose-dependent manner. It is also noteworthy that $1,25(OH)_2D_3$ reduces the synthesis and enhances the degradation of proteoglycans in MC-3T3-E1 cells [65, 66]. This is particularly relevant to the current study because two major classes of proteoglycan are remodeled during mineralization of bone [65, 67–69]. The first class is composed of aggrecan, the large aggregating proteoglycan. This proteoglycan endows cartilage and bone with its compressive properties, but is also an inhibitor of mineralization *in vitro* [24, 25, 29, 56, 57]. The second class is composed of decorin and biglycan, so-called small proteoglycans. Decorin is believed to regulate collagen fibrillogenesis and inhibit mineralization [70–73]. In our study we used aggrecan as the substrate in our enzyme assay and as a result we have confidence in our conclusion that these proteoglycans, which inhibit mineralization, are degraded by the matrix vesicle enzymes. In contrast, we do not have proof that decorin is degraded by any of the matrix vesicle enzymes, but the substrate specificity of metalloproteinases for leucine, isoleucine, or valine residues in the P1′ site and glycine or hydrophobic residues in the P1 site [74], coupled with a sequence of leucine-rich repeats in the decorin core protein amino acid sequence [68, 71], suggest that both large and small proteoglycans may be susceptible to the same or similar proteinases.

In summary, we have shown that osteoblast-like cells produce matrix vesicles containing metalloproteinases capable of digesting proteoglycan. We hypothesize that these proteinases are sequestered from the matrix until the membrane ruptures. In addition, the current study has shown that conditions favoring calcification *in vitro* produce matrix vesicles significantly enriched in metalloproteinases over those produced by cells without stimulation. Together these observations suggest that matrix vesicles may represent a novel mechanism whereby the cell can regulate mineralization, extracellular matrix remodeling, and possible proteinase-mediated activation of growth factors at sites distant from the cell surface.

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