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Matrix Vesicles Promote Mineralization in a Gelatin Gel

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Abstract. Extracellular matrix vesicles (MVs) are associated with initial calcification in a variety of tissues, but the mechanisms by which they promote mineralization are not certain. In this study, MVs isolated from fourth passage rat growth plate chondrocyte cultures were included within a gelatin gel into which calcium and phosphate ions diffused from opposite ends. In this gel, apatite formation occurs by 3.5 days in the absence of mineralization promoters, allowing measurement of the ability of different factors to ''nucleate'' apatite before this time or to assess the effects of molecules which modulate the rate and extent of mineral deposition. Mineral ion accumulation and crystal type are assayed at 5 days. In this study, MV protein content in the central band of a 10% gelatin gel was varied by including 100 μl of a Tris-buffered solution containing 0–300 μg/ml MV protein. There was a concentration-dependent increase in mineral accretion. Whereas 10 μg MV protein in the gel did not significantly promote apatite formation as compared with vesicle-free gels, 20 and 30 μg MV protein in the gel did promote apatite deposition. Inclusion of 10 mM β-glycerophosphate in the gels, along with MVs, did not significantly increase apatite formation despite the demonstrable alkaline phosphatase activity of the MVs. In contrast, MVs at all concentrations significantly increased apatite accumulation when proteoglycan aggregates or ATP, inhibitors of apatite formation and proliferation, were included in the gel. Slight increases in calcium, but not phosphate accumulation, were also noted when an ionophore was included with the MVs to facilitate Ca ion transport into the vesicles. FT-IR analysis of the mineral formed in the vesicle-containing gels revealed the presence of a bone-like apatite. These data suggest that MVs facilitate mineralization by providing enzymes that modify inhibitory factors in the extracellular matrix, as well as by providing a protected environment in which mineral ions can accumulate.

Key words: Mineralization *in vitro* — Matrix vesicles — Apatite — Proteoglycans — Chondrocyte.

Extracellular matrix vesicles (MVs) are associated with initial calcification in calcifying cartilage [1, 2], membranous bone [3], mantle dentin [4], and dystrophic calcification [5, 6], as well as in chondrocyte [7–9] and osteoblast [10–12] cultures. Although in many of these instances, MV and collagen calcification appear to be concurrent [9, 13], the processes may be distinct. The precise mechanism of MV-mediated calcification remains unclear, although studies using liposomes as MV models have indicated that apatite crystal formation is facilitated by providing a protected enclosure with high phosphate content (as may exist in MVs) into which calcium ions are transported [14–17]. It has been hypothesized that there is a specific interaction among the calcium ions, acidic phospholipids, and proteolipids in the liposome or MV membrane which facilitates apatite formation [18]. As the crystals form and proliferate within this liposome model of MV, they penetrate the liposome membrane, and then continue to grow in the extraliposomal environment [14–18].

It has also been suggested that MVs may facilitate mineralization by providing enzymes required for the mineralization process [19]. Enzymes such as alkaline phosphatase, Ca/Mg ATPase, and NTP-pyrophosphohydrolases can increase local phosphate concentrations and/or remove phosphate esters and pyrophosphates that inhibit mineral formation and growth [20]. Other enzymes, such as the metalloproteinases [19, 21, 22], can alter the composition and structure of the extravesicular matrix in such a way as to facilitate mineral crystal proliferation.

Preliminary data presented at the Fifth International Conference on Matrix Vesicles and Cell-Mediated Calcification [23] suggested that MVs at a concentration estimated to be that present in the growth plate could overcome inhibition of calcification by proteoglycan aggregates and ATP, but were unable to promote mineralization per se. Recent *in vivo* studies using a rat tibia marrow ablation model demonstrated that calcification of the healing bone correlated with a high density of MVs [24], indicating that concentrations of these extracellular organelles may be an important variable. In the present study, we extend our analysis of the role of action of MVs in the mineralization process by examining the ability of intact MVs to promote calcification in a gelatin gel system. This *in vitro* model, which requires only a small amount of isolated MVs, has previously been used to demonstrate that acidic phospholipids [25], low concentrations of phosphophoryn [26], biglycan [27], and bone sialoprotein [28] are mineralization promoters, whereas osteopontin [29], dentin sialoprotein [28], high concentrations of phosphophoryn [26], and aggrecan [25] are mineralization inhibitors.

Materials and Methods

Chondrocyte Cultures

The culture system used in the study has been described in detail elsewhere [30]. Rib cages were removed from 125 g Sprague-

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Dawley female and male rats under an Institutional Animal Care- and Use Committee-approved protocol and placed in Dulbecco's modified Eagle's medium (DMEM). The reserve zone (resting zone, RC) and growth zone (prehypertrophic and upper hypertrophic, GC) were separated, sliced, and incubated overnight in DMEM at 37°C in an atmosphere containing 5% CO_2 and 100% humidity. DMEM was replaced by two 20-minute washes in Hank's balanced salt solution (HBSS), followed by sequential incubations in 1% trypsin (Gibco Laboratories, Grand Island, NY) for 1 hour and 0.02% collagenase (Type II, Worthington Biochemical Corporation, Freehold, NJ) for 3 hours. All enzymes were prepared in HBSS. Cells were separated from debris by filtration through 40-mesh nylon, collected by centrifugation at 500 × *g* for 10 minutes, resuspended in DMEM, and plated in T75 flasks (Falcon, Becton, Dickinson Labware, NJ) at a density of 10,000 cells/cm^2 for resting zone cells or 25,000 cells/cm^2 for growth zone cells. Cultures were incubated in DMEM containing 10% fetal bovine serum (FBS) (Gibco) and 50 μg/ml vitamin C. Media were changed at 24 hours and then at 72-hour intervals. At confluence, cells were subcultured using the same plating densities and allowed to return to confluence. Fourth passage cells were used for these investigations, since prior studies had shown that differential response to vitamin D_3 metabolites is preserved at this passage [30–36].

Isolation of Matrix Vesicles

Matrix vesicles were isolated from chondrocyte cultures by differential centrifugation of trypsin digests of the cell layer [30, 32]. At harvest, the conditioned media were decanted and the cultures trypsinized (1% in HBSS). The reaction was stopped with DMEM containing 10% FBS, and the cells were separated from the digest supernatant by low-speed centrifugation. The digest supernatant was further centrifuged for 10 minutes at 13,000 × *g* to pellet a fraction containing mitochondria, large membrane fragments, and other cellular organelles, and for 1 hour at 100,000 × *g* to pellet the matrix vesicles. Plasma membranes were isolated from homogenates of the cell pellet by differential and sucrose density centrifugation [30, 32]. Matrix vesicles and plasma membranes were stored resuspended in 1.0 ml saline, and 100 μl were reserved for determination of protein content and alkaline phosphatase activity. The protein content of the MV and plasma membrane preparations was determined using the method of Lowry et al. [37]. Alkaline phosphatase specific activity of each membrane preparation was assayed [38] to ensure that MVs exhibited greater than a twofold enrichment over their respective plasma membrane fractions [30–36].

Most assays performed in this study used MVs isolated from growth zone chondrocyte cultures due to the greater numbers of MVs that could be isolated from these cultures in comparison with reserve zone chondrocyte cultures (8.5 ± 0.3 versus 3.9 ± 0.7 μg protein/μl membrane suspension, GC versus RC), as well as the higher alkaline phosphatase specific activity of the MVs produced by the growth zone cells (123.0 ± 14.0 versus 40.5 ± 5.1 μmole P_i/mg protein/minute, GC versus RC). Following determination of protein content and alkaline phosphatase activity, the MVs were pelleted by centrifugation at 100,000 × *g* and transported on dry ice. Prior to calcification assays, samples were resuspended in the appropriate solutions and gels as described below, based on protein content.

Demonstration That Matrix Vesicles Can Accumulate Calcium

To verify that the MVs isolated from culture and used in the mineralization assays in the present study behaved similarly to those isolated directly from tissues with respect to calcium accumulation, the methodology described by Valhmu et al. [39] was followed. Suspensions of MVs containing a final concentration of 40 μg protein/ml were incubated in 2 ml of synthetic lymph, consisting of equal volumes of solution A (102.7 mM NaCl, 10.3 mM KCl, 4.7 mM KH_2P0_4, 9.5 mM K_2HP0_4, 5.7 mM $MgCl_2$, 55.5 mM glucose, and 635 mM sucrose) and solution B (2 mM $CaCO_3$ in 18 mM Tris buffer at pH 7.5). ^{45}Ca (5 μl of 1 μCi/ml) was included in each experiment. Controls were prepared to have the same solution concentrations and ^{45}Ca activity, but contained no MVs. At selected time points, 100 μl aliquots were removed, and any mineral associated with the MVs was collected by filtration onto 0.45 μm Millipore filters using a Millipore 1225 sampling unit (Millipore, Inc., Philadelphia, PA). Dry filters were mixed with Aquasol scintillant and the time-dependent ^{45}Ca accumulation was monitored. Each time point was sampled in duplicate, and results from four separate experiments with equivalent concentrations of MVs were combined. In some experiments, ^{45}Ca was excluded and the Ca content was determined by flameless atomic absorption using a Perkin Elmer model 5500 spectrometer (Norwalk, CT). For these analyses, the filters were hydrolyzed in 1 N HNO_3.

Mineral Formation in a Gelatin Gel

Mineralization was monitored in a double diffusion system [25], in which Tris-buffered Ca^{2+} (100 mM) and $HPO_4^{=}$ (100 mM) ions flow separately from opposite directions into a 10% gelatin gel (Bloom gelatin, Fisher Chemical, Springfield, NJ) contained in 6-cm segments of 10 cc polystyrene disposable tubes (Fisher Chemical). In the absence of nucleators, promoters, or inhibitors, apatite formation occurs 1.35 ml from the Ca entry side when the Ca × PO_4 concentration reaches 5.5 mM^2. This occurs approximately 3.5 days after the diffusion of fluids is begun. Changes in the rate or amount of Ca and phosphate (P) accumulated at the precipitant line when materials are incorporated into the gel indicate whether the materials are nucleators, promotors, or inhibitors of apatite formation, crystal multiplication, and crystal growth. If there is an increase in accumulated mineral, the materials are promotors, and may be nucleators. A delay in the formation of apatite or a decrease in the amount of mineral accumulated at the precipitant line is an indication that the incorporated materials are inhibitors.

Matrix vesicles were included in 100-μl bands at the site of the future precipitant band by mixing 50 μl of a 2× MV suspension or buffer (control) with 50 μl 20% gelatin and sandwiching the mixture between two layers of 10% gel. The resultant MV bands contained 10, 20, or 30 μg MV protein. Gels contained 0.01% sodium azide or 1% of a penicillin/streptomycin stock containing 10 mg/ml penicillin and 10 mg/ml streptomycin sulfate (Gibco) to prevent bacterial growth. The gels were mounted on an apparatus that circulated the external solutions at a constant rate [29]. Each experiment had six types of gels, each in triplicate (double-diffusion experimental and control gels, and Ca and PO_4 single-diffusion experimental and control gels), for assessment of ion accumulation due to diffusion or entrapment in the MVs. Each type of experiment was repeated at least three times. Experiments were used to address questions related to the proposed mechanism of action as indicated below.

Gels were monitored visually to determine whether a precipitant band, indicating mineral formation, was detectable earlier than 3.5 days, and the mineral yields at 3.5 and 5 days were determined. In some experiments, the mineral content of the precipitant band was analyzed by wide angle X-ray diffraction using CuKα radiation or by FT-IR spectroscopy of KBr pellets. For the diffraction studies, excess gelatin was removed by melting the gel at 55°C. For FT-IR, 200 μg of lyophilized material was mixed with 100 mg of KBr and spectra recorded on a Mattsen spectrometer (Madison, WI) at 4 cm^{-1} resolution, 256 scans per spectrum. For analysis of Ca and P accumulation, gels were sliced into 0.3 cm bands, with the precipitant band at the center; the slices were hydrolyzed in 1 N HCl and the Ca [40] and P [41] content were measured. The amount of mineral accumulated was calculated as the difference between double-diffusion and single-diffusion gels. Results were always compared to matrix vesicle-free controls run in the same experiment.

To determine whether the ability of MVs to promote apatite formation in the gelatin gel model was dependent on the state of

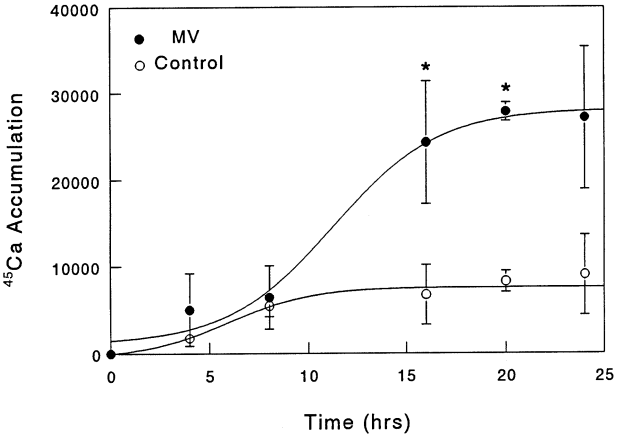

Fig. 1. ^{45}Ca accumulation by matrix vesicles (MV) suspended in synthetic lymph. Aliquots were withdrawn at indicated times. Controls received an equivalent volume of buffer in place of MVs. Values are mean ± SD for three separate experiments, each of which included three vesicle preparations per time point and three controls per time point. *Significantly different from control at same time point, $P \leq 0.05$, based on Bonferroni's multiple comparisons test.

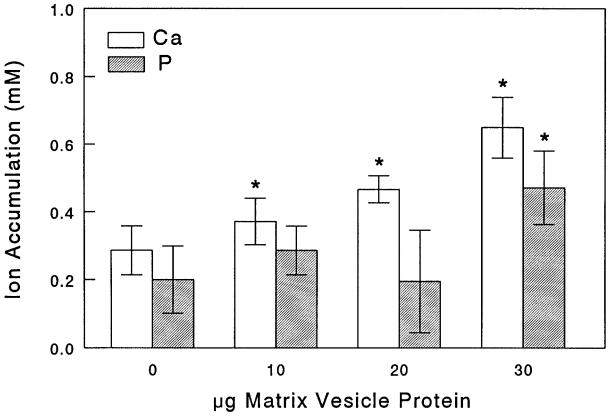

Fig. 2. Ca and P content of the precipitant band in gelatin gels containing MVs. Matrix vesicle protein content was varied from 0 to 30 μg/gel. Samples were incubated for 5 days. Data are corrected for ion accumulation in single diffusion gels. *Significantly different from matrix vesicle-free control (0 μg protein), $P \leq 0.05$, based on Bonferroni's multiple comparisons test.

chondrocyte maturation *in vivo*, MVs isolated from both resting zone and growth zone chondrocyte cultures were examined.

To assess the role of MV phosphatases, the entire gel contained either 10 mM β-glycerophosphate (βGP) or the central band contained 1–3 mM ATP with or without MVs. The pH of the gel was readjusted to 7.4 after mixing with βGP or ATP and before adding the MVs. All other conditions and assays were as above. In addition, to determine if the mixing of the MVs with gelatin at 42°C had affected the MV alkaline phosphatase activity, some gelatin slices were stained for alkaline phosphatase activity using L-naphthol phenyl phosphate as substrate [42].

To test the hypothesis that MVs are important in removing proteoglycan inhibitors surrounding them *in situ*, proteoglycan aggregate was included in the gelatin gel. The proteoglycan A1A1 fraction from bovine nasal cartilage [25] was kindly provided by Dr. Lawrence Rosenberg (Montifiore Hospital, Bronx, NY). The proteoglycan was dissolved in Tris buffer at 40°C at 4× concentration, mixed with MVs, and then with 20% gelatin. Controls were matrix vesicle-free. In some cases, the proteoglycan-MV mixture was incubated with stirring at 37°C for 24 or 72 hours. Controls included 4 or 10 ng/nl proteoglycan aggregate. Analyses were as above.

To investigate the possibility that isolation of the MVs resulted in the loss of the ability for Ca transport, the vesicles were first suspended in a 1 mM $CaCl_2$ solution containing 10 μl of a 6 μM solution of the Ca ionophore, A23187 (Sigma, St. Louis, MO), in 90% ethanol. Controls received a comparable amount (10 μl) of ethanol, MVs, and 1 mM Ca. Analytical methods were as detailed above.

Results

Matrix vesicles isolated from growth zone chondrocyte cultures behaved in solution (Fig. 1) comparable to those studied by Valhmu et al. [39]. Accumulation of ^{45}Ca increased with time, and after 8 hours was, in general, significantly greater ($P \leq 0.05$) than control. The large scatter reflects experiment-to-experiment variation, due perhaps to the different MV preparations used. Measurement of total Ca accumulation in the absence of ^{45}Ca failed to show significant differences when experimental (matrix vesicle) and control (MV-free) solutions were compared (data not shown).

Matrix vesicles caused a concentration-dependent increase in the Ca and phosphate content of the gelatin gel precipitant band at day 5 (Fig. 2). Ca accumulation was significant at the lowest concentration of MVs, whereas phosphate accumulation was significant only in gels containing 30 μg MV protein in the central band. No significant differences were noted between control gels and gels containing MVs at 3.5 days. Matrix vesicles isolated from resting zone chondrocyte cultures at 10 μg/gel had no effect on Ca and P accumulation; however, at 20 and 30 μg/gel, they behaved comparably to MVs isolated from growth zone chondrocyte cultures (data not shown).

The mineral formed at the precipitant line in all cases was apatite, with crystallite size and perfection similar to that in bone apatite, as demonstrated by X-ray diffraction (not shown) and FT-IR (Fig. 3).

βGP had no effect on Ca on P accumulation in the precipitant band, whether or not MVs were present (Fig. 4). When 10 μg MV protein was included, there was little effect on the mineral accumulation in the presence of 10 mM βGP. At 3.5 days, MV and control values were equivalent. At 5 days, MV values were slightly, but not significantly, greater than controls despite the alkaline phosphatase activity detectable in the slices of the gels containing the vesicles (Fig. 5).

Inclusion of ATP resulted in a dose-dependent decrease in Ca accumulation in the matrix vesicle-free controls (Fig. 6). A similar trend in P accumulation was noted, but the effect was not statistically significant at the times assayed. When MVs were included, Ca and P accumulation increased, both as a function of ATP concentration and as a function of time.

Matrix vesicles overcame the inhibition of calcium and phosphate accumulation caused by proteoglycan (Fig. 7). The effect was independent of MV concentration, although a trend was noted indicating maximal effect between 10 and 20 μg matrix vesicle protein/gel. It was not necessary to preincubate the MVs with proteoglycan to see an effect. However, when the MVs were preincubated for 24 hours at 37°C with either 10 mg/ml proteoglycan (Fig. 8) or 4 mg/ml proteoglycan (data not shown), there was an increase in the ability of the MVs to overcome the proteoglycan-dependent inhibition. At 3.5 days, controls contained negligible amounts of mineral, making it difficult to evaluate the gels.

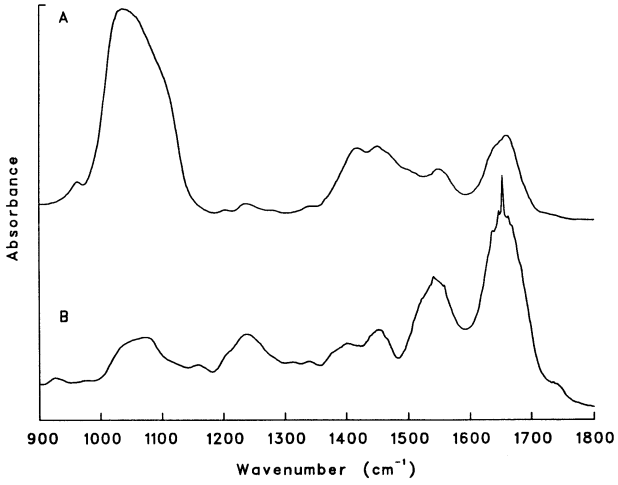

Fig. 3. FT-IR analysis of a gel containing MV and proteoglycan at 3.5 days (**B**) contrasted with mouse bone (**A**). The mineral is indicated by the broad band at 900–1200 cm^{-1}, the protein (collagen, or denatured gelatin, respectively) by the bands at 1500–1800 cm^{-1} in the MV-containing gel which may be due to both proteoglycan and lipid.

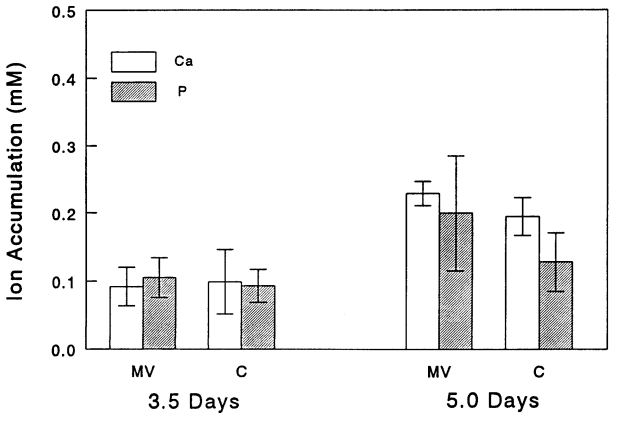

Fig. 4. Effect of β-glycerophosphate (βGP) on the ability of MV to promote Ca and P accumulation in the precipitant band of gelatin gels. All gels contained 10 mM βGP. MV (10 μg protein/gel) were included in ½ of the gels. Ca and P accumulation were measured at 3.5 or 5.0 days. Data are corrected for ion accumulation in single diffusion gels. βGP had no effect on accumulation of either ion at either time point, whether or not MV were present.

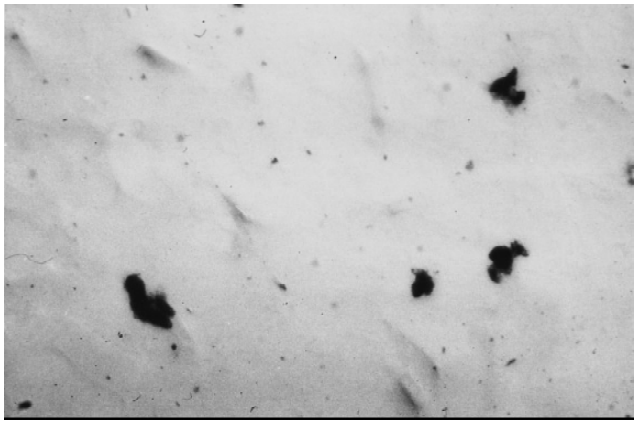

Fig. 5. Photomicrograph of gelatin slices stained for alkaline phosphatase activity using L-naphthol phenyl phosphate as a substrate.

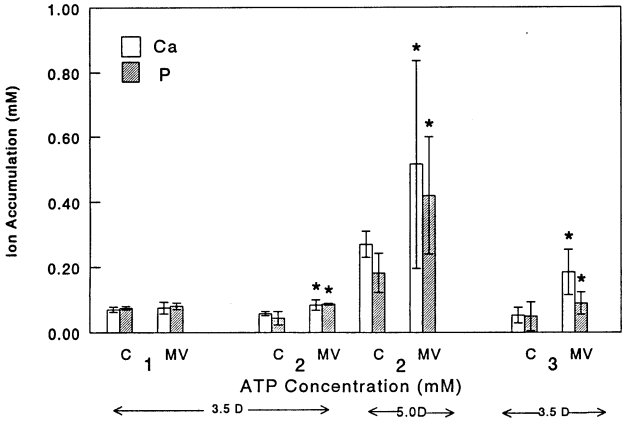

Fig. 6. Effect of ATP on the ability of MV to promote Ca and P accumulation in the precipitant band of gelatin gels. Gels contained 1, 2, or 3 mM ATP. MV (10 μg protein/gel) were included in ½ of the gels. Ca and P accumulation were measured at 3.5 or 5.0 days. Data are corrected for ion accumulation in single diffusion gels. *Significantly different from matrix vesicle-free control (0 μg protein), $P \leq 0.05$, based on Bonferroni's multiple comparisons test. For C2 cultures (2 mM ATP), Ca and P accumulation at 3.5 days was less than that at 5 days (Ca: $P < 0.001$; P: $P < 0.05$). C1 versus C2 versus C3 was not significantly different. However, when testing for a linear trend by linear regression, the Ca values were significant ($P < 0.0001$) but P values were not.

Incubation of the MVs with a Ca ionophore slightly increased calcium ion accumulation relative to untreated matrix vesicles (Fig. 8), but had no effect on phosphate ion accumulation. The mineral formed in the presence and absence of the ionophore appeared similar by FT-IR spectroscopy (data not shown).

Discussion

Extracellular MVs have long been associated with the initial deposition of mineral crystals in calcified tissues [1–8]. The present study, performed using MVs derived from culture in a gelatin gel assay system, sheds new light on how these organelles may function *in vitro,* and by analogy, *in vivo.* Data presented show that MVs isolated from chondrocyte cultures behave in solution like MVs derived from cartilage with respect to ^{45}Ca accumulation [20, 39]. In addition, they promote calcium and phosphate accumulation in gels in at least three different ways. At sufficiently high concentrations, MVs can increase Ca and phosphate accumulation, indicating that they may as a ''nucleator.'' At low concentrations, they may contribute to calcification by removing or modifying inhibitors both within the vesicle, like ATP, and in the matrix, like proteoglycan.

This suggests that an important function of the MV is to provide enzymes that modulate the extravesicular tissue composition and enzymes that alter internal vesicle compo-

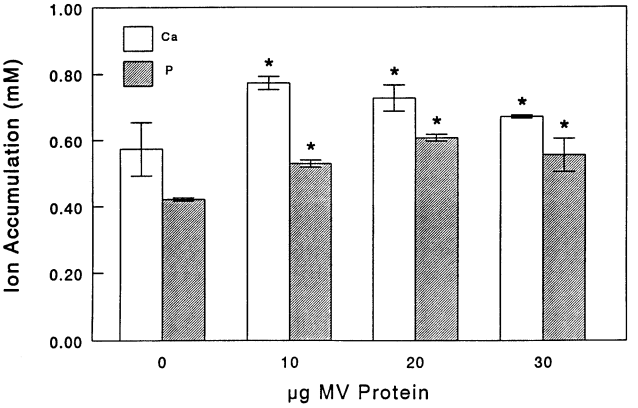

Fig. 7. Ability of MV to promote Ca and P accumulation in the precipitant band of gelatin gels containing 10 mg/ml proteoglycan (aggregan). Gels contained 0, 10, 20, or 30 μg MV protein. Ca and P accumulation were measured at 5.0 days. Data are corrected for ion accumulation in single diffusion gels. *Significantly different from matrix vesicle-free control (0 μg protein), $P \leq 0.05$, based on Bonferroni's multiple comparisons test.

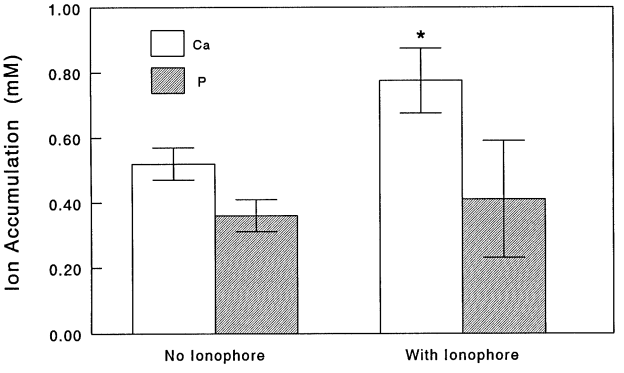

Fig. 8. Effect of the ionophore A23185 on the ability of MVs (20 μg protein/gel) to accumulate Ca and P in the precipitant band of gelatin gels. Gels were incubated for 5.0 days. Data are corrected for ion accumulation in single diffusion gels. *$P < 0.05$, + ionophore versus no ionophore, based on Bonferroni's multiple comparisons test.

sition. Since the presentation of these enzymes can be modulated by hormones and growth factors [22, 33, 35, 43, 44], this mechanism may account for many aspects of the cell-mediated mineralization process *in vivo.* It is important to note that the model used in this study results in only an approximate analogy, since the gelatin is denatured type I collagen, whereas cartilage contains type II collagen and ionic concentrations increase continuously rather than being maintained at constant values. Moreover, *in vivo* the composition and activity of MV components can be modulated by the cells not only during MV production but also through nongenomic mechanisms once the organelles are in the matrix [33, 34, 44], which may further affect the rate and extent of mineral that can form.

Despite these caveats, the results provide valuable information concerning the mechanisms by which MVs may promote mineralization *in vivo.* Matrix vesicle concentration appears to be an important variable, since lower concentrations did not enhance Ca and P accumulation in the gels, whereas at higher concentrations, they did. Models by which vesicles can sequester ions, providing a protected environment for the formation of the first crystals, have been described in detail elsewhere [14–18, 45]. Increasing the concentrations of liposomes in solution has been shown to result in an increased yield of mineral [46]. Thus, it is not surprising that, as the concentration of MVs was increased in the gels, the yield of mineral increased. In tissues, where MVs may increase in concentration at sites of new mineral deposition [24], they may function in this way.

The rat chondrocyte culture-derived MVs accumulated ^{45}Ca in a manner comparable to that reported for MVs isolated directly from bovine [47–49] or chicken [39, 50, 51] growth plate cartilage. Some of the ^{45}Ca may have been associated with the outer membrane of the MVs and the contribution of this to the overall uptake was not addressed in the study. Although ^{45}Ca accumulation is not synonymous with apatite formation, the ability of MVs to do so supports the hypothesis that they are capable of mineral accretion.

Several studies have shown that MVs from calcifying cartilage contain proteins associated with ion transport across membranes: annexin V [50] and proteolipids [53]. Both annexin V [50] and proteolipid [53], as well as calcium-phospholipid-phosphate complexes [52], function as ''nucleators'' when incubated in metastable calcium phosphate solutions [54]. Similarly, Wu et al. [51] have described a ''nucleational core'' complex present within MVs which consists of proteolipid, matrix proteins, and complexed acidic phospholipids. This suggests that these MV components may contribute not only to calcium accumulation but also to apatite nucleation in the gelatin gel model.

Whether the increased mineral accumulation was due to nucleation per se is not clear. Nucleation is a physicochemical process through which ions or ion clusters accumulate with the correct orientation to mimic a crystal surface. Heterogeneous nucleators provide a surface that matches that of the substrate in question. Lipids have been shown to be nucleators of glycine and sodium chloride crystals [55] and are proposed as nucleators of apatite formation [56]. However, since the detailed process of acidic phospholipid-induced apatite deposition has not been demonstrated to be one of ''nucleation,'' it can only be suggested that lipids, specifically lipids within MVs, promote calcification.

The MVs used in the present study exhibited enrichment of alkaline phosphatase specific activity typical of MV isolated from tissue. Furthermore, this enzyme remained functional in the gel. Thus, it was puzzling not to see enhanced mineral formation in the presence of βGP. One possibility is that at the high concentration used, 10 mM, βGP acted as an alkaline phosphatase inhibitor due to a local increase in phosphate concentration. Such inhibition has been noted in several other systems [54].

In contrast to experiments with 10 mM βGP, when lower concentrations of ATP were included in the gels, the MVs were able to effectively utilize it as a phosphatase substrate. In gels with MVs, the apatite inhibitor was removed and Ca and P accumulation returned to normal control levels. Vesicles are known to have ATPase activity [20, 57], an activity important because it can reduce the concentration of ATP, perhaps both inside and outside the vesicle. ATP is an inhibitor of apatite crystal formation and growth, whereas its hydrolytic products (ADP and AMP) have little effect on this process [58]. Further, hydrolysis of ATP would increase the local phosphate concentration, facilitating mineral proliferation. At the higher ATP concentrations, MVs used the

released phosphate to enhance apatite formation. Thus, in the presence of calcification inhibitors, even a low concentration of MVs can promote mineral deposition through action of its enzymes, whether or not the organelle acts as a classical nucleator.

This hypothesis was supported by the ability of the MVs to reverse the proteoglycan-dependent inhibition of apatite formation in the gels. Matrix vesicles isolated from tissue contain neutral peptidases [21]. Similarly, the MVs used in the present study contain neutral metalloproteases [19], including stromelysin [43], which are capable of degrading proteoglycans. Activity of these enzymes is vitamin D-dependent [35, 59], suggesting a mechanism by which this hormone can regulate cartilage calcification. By degrading or modifying the inhibitory proteoglycans in the extracellular matrix, MVs could facilitate the spread of apatite crystals.

Formation of initial mineral crystals in the vesicles, both in liposome model systems [14–18] and in the gel system studied here, may be stimulated when Ca transport into the vesicles is facilitated. This is in agreement with the view that a calcium transporter, annexin V, plays an important role in initiating vesicle calcification [50, 51].

The mineral associated with the MVs throughout these studies was apatitic, comparable to the mineral found in bone in terms of crystal size and perfection. This is distinct from the amorphous calcium phosphate [60] reported to be present in isolated vesicles or the octacalcium phosphate-like phase reported to be formed when MVs are incubated in synthetic lymph [60]. Since the preparations in this study were examined only after initial mineral was visible and since Sauer and Wuthier [61] also reported a transition of octacalcium phosphate in MVs to apatite, the present results do not eliminate the possibility that other phases served as precursors; rather, they demonstrate the usefulness of the gel system for these types of analyses.

In conclusion, results of this study support the hypothesis that MVs can promote initial apatite formation by (1) providing a protected site for initial mineral formation; (2) presenting potential nucleators (not studied here) within the vesicle to serve as sites for initial crystal formation; (3) providing enzymes and other activities that can increase the flux of ions into the vesicles; and (4) providing enzymes which modify the composition of the extracellular matrix. The last may be the most important, as it would allow crystals to spread into the matrix.

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