American Journal of Pathology, Vol. 136, No. 2, February 1990

Matrix Vesicle Biogenesis *In Vitro* by Rachitic and Normal Rat Chondrocytes

H. C. Anderson, D. J. Stechschulte, Jr., D. E. Collins, D. H. Jacobs, D. C. Morris, H. H. T. Hsu, P. A. Redford, and S. Zeiger

From the Department of Pathology and Oncology, University of Kansas Medical Center, Kansas City, Kansas

Calcifying matrix vesicles (MVs) are released from chondrocytes and osteoblasts in monolayer culture. In the present studies, we tested the ability of rachitic* versus *normal rat growth plate chondrocytes in micromass or monolayer primary cultures to produce MVs. Unlike earlier reports of* in vitro *MV biogenesis by chicken chondrocytes in which most MVs were released into the medium, we found that most of the released rat matrix vesicles were entrapped in a newly formed cartilaginous matrix enveloping the cells. These matrix-associated MVs could be isolated by mild collagenase treatment and concentrated by differential centrifugation. Vesicle production slowed in the older 2- to 4-week-old cultures and, unlike vesicle release from cultured chicken chondrocytes, active vesicle production did not show a second burst of activity at 3 to 4 weeks. Alkaline phosphatase (ALP) activity diminished with time in culture in cells and matrix vesicles, suggesting a decrease in differentiative expression. Protein profiles on SDS polyacrylamide gels of native matrix vesicles and culture-derived MVs from rachitic and normal cells were quite similar and showed a typical simplified protein pattern as compared to chondrocyte plasma membrane proteins. There were distinctive proteins migrating at 130, 80 to 95, 66, 43, 20, and 14 kd. Culture-derived MVs showed vigorous* in vitro *calcifying activity that was ALP related. We conclude that 1) rachitic chondrocytes are essentially normal in their matrix vesicle production; 2) matrix entrapment of MVs is a characteristic of rat chondrocyte cultures; and 3) culture-produced MVs are similar to native MVs in protein profile and calcifiability, and thus can be studied as a model for normal MV composition and calcification. (Am J Pathol 1990, 136: 391–398)

In cartilage, bone, and dentin, biologic calcification is initiated within and at the surfaces of extracellular matrix vesicles (MVs). The latter are derived from adjacent osteoblasts, chondrocytes, and odontoblasts.[1–12] Earlier work indicates that matrix vesicles initiate mineral deposition through the action of MV-associated phosphatases[13–16] and calcium-binding phospholipids[16–20]; however, little is known about the mode and regulation of matrix vesicle biogenesis.

Several mechanisms of biogenesis have been proposed,[21] including 1) pinching-off from the cell surface[1,2,6,7,21]; 2) cell fragmentation (apoptosis)[1,6,22]; 3) extrusion of preformed vesicles through the outer cell membrane[1,12,23]; and 4) *de novo* aggregation of molecular substituents of membranes in the extracellular space to form liposome-like vesicles.[21,24] Recently, it has been proposed that matrix vesicles may break up and fuse with each other as they approach the calcification front to form larger vesicles.[25]

In an attempt to analyze in greater detail the mechanism of MV production by cells, culture systems were devised in which chicken limb and/or growth plate chondrocytes, cultured in monolayer, released MV-like vesicles into the culture medium.[16,26,27] Such MVs could be isolated from the culture medium by simple differential centrifugation. The main findings of these earlier studies were that 1) copious vesicles were released into the medium[16,26,27]; 2) their phospholipid composition resembled that of native mammalian matrix vesicles,[16,26] and to some extent that of the plasma membrane of the producer chondrocytes; 3) MVs formed in culture possessed a strong Ca^{2+}-affinity[16,26,27] that could be boosted by adding 5mM Ca, 5mM Mg, and 5mM ATP to the *in vitro* calcification medium[27]; and 4) chondrocyte cytoskeleton appears to be involved in the budding off of MVs because cytoskeletal modifiers such as cytochalasin D, phalloidin, and colchicine can alter the rate of MV formation.[28]

In parallel experiments, we investigated MV production by mammalian, rachitic rat growth plate chondrocytes using, at first, micromass cultures[29,30] and then comparing

Accepted for publication October 2, 1989.

Address reprint requests to H. Clarke Anderson, MD, Department of Pathology and Oncology, University of Kansas Medical Center, 39th and Rainbow Boulevard, Kansas City, KS 66103.

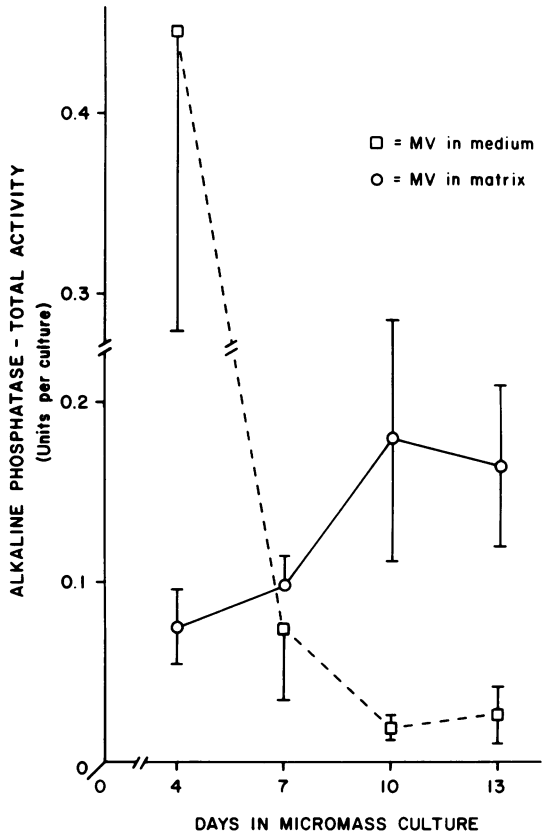

Figure 1. *Changes in total vesicle-associated alkaline phosphatase in medium (□) or associated with matrix (○), during 2 weeks in micromass culture of rachitic chondrocytes. The sedimentable (vesicle-associated) ALP in the culture medium declined to very low levels during the first week, while the collagenase-released sedimentable ALP associated with the cells rose moderately during the second week (vertical bars represent ± standard error).*

micromass and monolayer cultures.[29] Parallel studies were carried out by Boyan et al[31] and Schwartz et al[32] using normal rat costochondral chondrocytes. It was found that MVs are produced by mammalian chondrocytes[29-32] and that they calcify *in vitro* to form apatitic deposits.[30,33] However, unlike MVs produced by avian cell cultures, rat MVs became entrapped in a newly formed cartilaginous matrix and few were released to the culture medium, regardless of whether monolayer or micromass techniques were used.[29] We also have found that mammalian MVs formed *in vitro* can be removed from their matrix entrapment by mild collagenase digestion and differential centrifugation without loss of structure or function.

The studies reported here were undertaken to further characterize the MVs produced by cultured mammalian chondrocytes with regard to 1) kinetics of formation in short- and long-term monolayer and micromass cultures; 2) calcifiability; and 3) protein content as demonstrated by polyacrylamide gel electrophoresis. In general, the results support the assumption that rachitic growth plate chondrocytes do not differ materially from normal MVs insofar as MV production, calcification, and protein content are concerned.

Materials and Methods

Cell Isolation and Culture Methods

Growth plates were removed sterilely from normal and rachitic weanling Sprague Dawley rats. Rickets was induced by housing 15-g weanling rats in a darkened room and feeding them a diet low in phosphate and vitamin D.[34] The result is growth retardation,[34] enlargement of the cartilaginous growth plates,[35] retarded mineralization of growth plates, and metaphyses of long bones.

At removal, the growth plates were minced and incubated for 3 hours in a solution containing 1000 units/ml of crude collagenase solution[36] at 37 C with constant rocking agitation. After 3 hours, the mostly liquified collagenase suspension was centrifuged at 1000 RPM for 10 minutes to remove cells and larger cell debris, at 14,000*g*

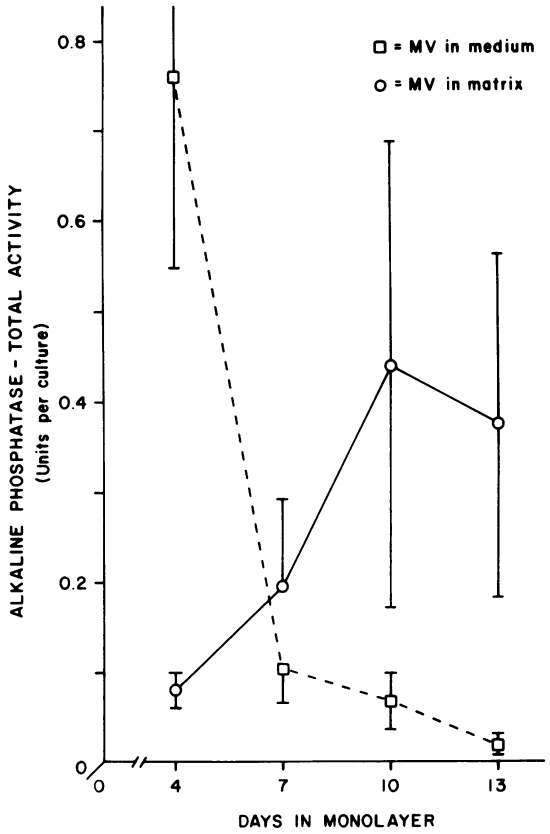

Figure 2. *Changes in total vesicle-associated ALP in medium (□) or associated with cell matrix (○) during 2 weeks in monolayer culture of rachitic chondrocytes. Although the variation in this data is greater than in Figure 1, the same pattern of decreasing particulate ALP in the media and accumulation of particulate ALP in cell-matrix occurs in monolayer as in micromass cultures.*

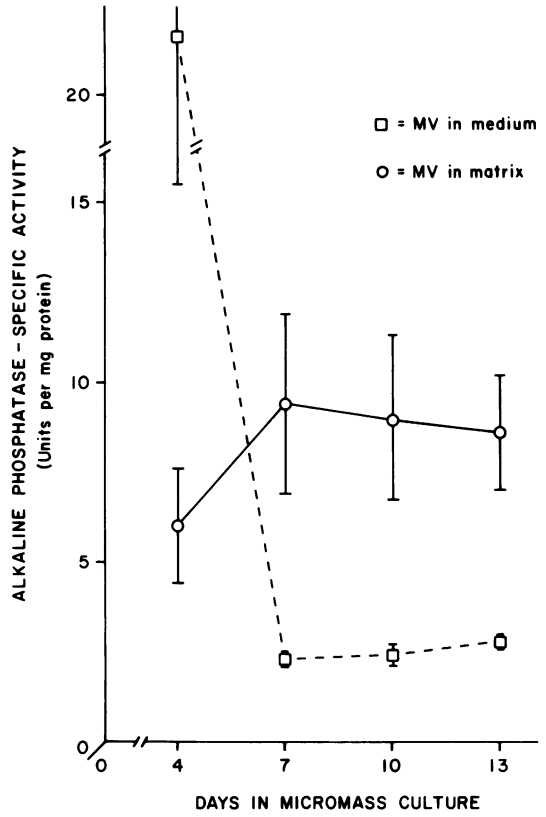

Figure 3. *Changes in vesicle-associated specific ALP activity in the medium (□) or in collagenase digested cell-matrix (○) during 2 weeks in micromass culture of rachitic chondrocytes. As with total ALP activity, the specific activity in the media declines to a very low level in 1 week, while specific activity rises in vesicles that are sequestered in the cell-associated matrix.*

for 30 minutes to remove nuclei, mitochondria, lysosomes, and small cell fragments, and at 300,000*g* for 30 minutes to sediment native matrix vesicles.[36,37] Cell counts revealed approximately 15 × 10^6 cells per gram of normal or rachitic growth plate cartilage with usually more than 90% viability as indicated by trypan blue exclusion.

Under standard conditions, 2 × 10^6 chondrocytes were plated per 35-mm culture dish as monolayers or micromass aggregates[30] in BGJb medium plus 10% fetal calf serum and maintained at 37 C in a humidified atmosphere of 5% CO^2 in air. The medium was changed three times per week. Confluence was reached at about 5 days. Usually the cultures were harvested at 7 to 14 days, but in one series of experiments, cultures that had been seeded at varying densities were maintained for up to 28 days before harvesting.

Matrix Vesicle Isolation

At the time of harvest, the culture medium was removed, the cultures were washed gently in fresh BGJb medium plus 10% FCS, then in one-half strength collagenase solution (500 units/ml) for 10 minutes and scraped into sterile roller tubes for agitation on the rocker at 37 C for 90 minutes in one-half strength collagenase solution. The resulting liquid suspension of cells and matrix-associated MVs was centrifuged according to the above scheme to separate cells, cell fragments and nuclei, and finally MVs. The culture supernates collected at times of feeding were centrifuged twice to remove cell fragments and then at 300,000*g* for 30 minutes to precipitate vesicles from the culture medium (ie, media vesicles). All vesicle pellets were resuspended for one wash in tris-buffered saline, reprecipitated at 300,000*g*, resuspended in TBS, and stored at −70 C before testing.

The presence and concentration of MVs in the 300,000*g* sediments was estimated by measuring alkaline phosphatase (E.C.3.1.3.1.)[37] and relating ALP activity to total protein,[38] ALP being a good marker for matrix vesicles.[36,39] For electron microscopic examination, the MV pellets were fixed in 2.5% glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated, embedded in Spurr's low viscosity epoxy resin, cut with diamond knives, and stained with uranyl acetate and/or lead citrate.[2] Thin (50 nM) sections were examined and photographed in a Zeiss EM10A electron microscope (Carl Zeiss, Oberkochen, FRG).

In Vitro *Calcification Assessment*

Calcifiability was assessed by a modification of the method of Hsu and Anderson.[37] Matrix vesicle fractions

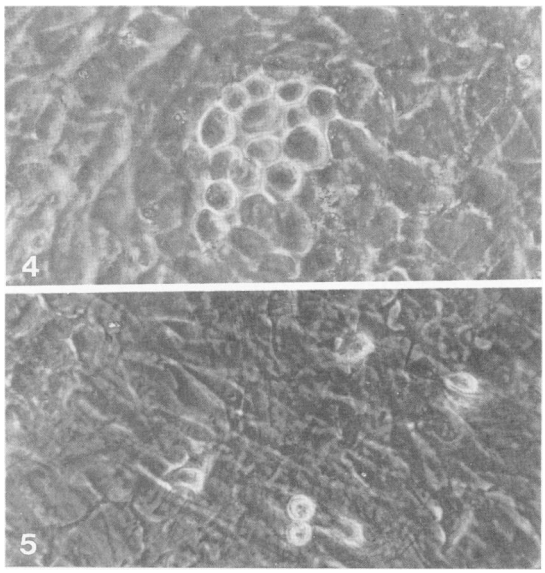

Figure 4. *Seven-day monolayer culture of rachitic rat chondrocytes, seen in phase-contrast. Small clusters of cells are beginning to be surrounded by a lucent extracellular layer of matrix that appears as a halo around the cells.* × *200.* Figure 5. *Twenty-seven day monolayer culture of rachitic rat chondrocytes, seen in phase-contrast. The cell layer is dense and apparently overlapping. Cells with matrixlike halos are less numerous and more widely dispersed.* × *200.*

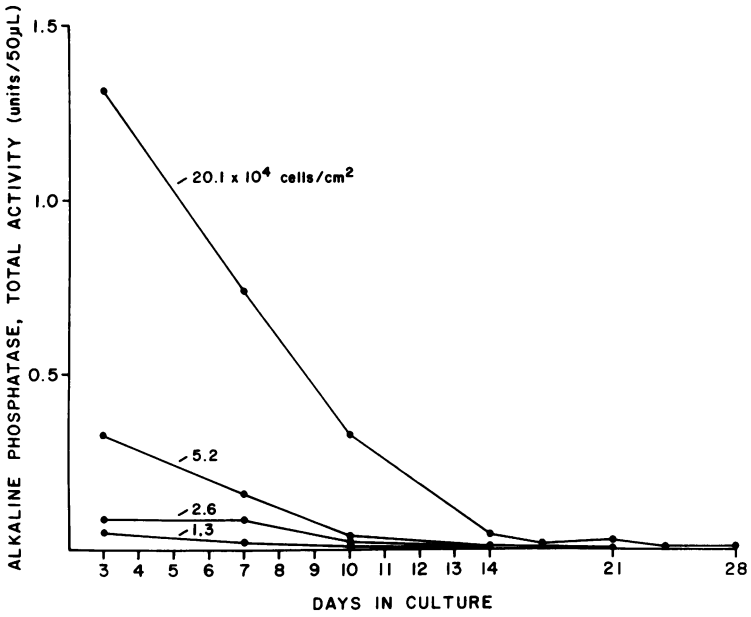

Figure 6. *The release of particulate (vesicle) total ALP into the medium declines to nondetectible levels within 2 weeks in long-term monolayer cultures of rachitic rat chondrocytes seeded at differing cell densities from 1.3 × 10^4 cells/cm^2 to 20.1 × 10^4 cells/cm^2. The amount of particulate ALP released is directly related to the initial seeding density.*

containing 0.18 units of alkaline phosphatase (0.36 units in the case of media vesicles) were exposed to a calcifying solution (2.2 mM $CaCl_2$ containing 100,000 cpm ^{45}Ca, 1.6 mM KH_2PO_4, 1.0 mM $MgCl_2$, 85 mM NaCl, 15 mM KCl, 10 mM $NaHCO_3$, and 50 mM TBS) for 5.5 hours at 37 C and pH 7.6. These experiments were carried out in tandem with one half of each group receiving 3.0 mM AMP as ALP substrate and the others an equal amount of 50 mM TBS diluent to maintain a constant volume of 250 μl in each reaction vessel. After incubation, the reaction vessels were centrifuged at 8800*g* for 30 minutes to precipitate calcified vesicles, and the precipitate was washed twice with 0.5 ml chilled TBS, followed by resedimentation at 8800*g*, and finally resuspended in scintillation fluid[37] for counting of ^{45}Ca in the precipitate as an indication Ca deposition by the vesicles.

Protein Analysis

MV protein (91 to 100 μg) in sodium dodecyl sulfate was electrophoresed in 7.5% to 10% polyacrylamide gels by the method of Laemmli[40] and stained with coomassie brillant blue.

Results

Pattern of Matrix Vesicle Production

During the first 2 weeks of cultivation of normal and rachitic growth plate chondrocytes in micromass or monolayer cultures, the number of sedimentable MVs in the media declined, but those in the collagenase-dissociable matrix increased (as reflected by total particulate ALP) (Figures 1, 2). This inverse relationship in vesicle appearance in medium *versus* matrix was also discernible as inverse changes in specific activity of particulate ALP (Figure 3). Although not depicted, the same pattern of vesicle accumulation in matrix and decline of vesicle release into medium was observed in cultures of nonrachitic chondrocytes.

In long-term primary cultures of up to 4 weeks, the chondrocytes appeared intact by phase-contrast microscopy (Figures 4, 5); however, ALP-positive vesicle release into the medium did not resume after the initial day 3 to 5 release (Figure 6). Specific activity of ALP declined in the media-derived vesicles after an initial twofold increase at 7 days. The failure of a late release of MVs into the medium occurred regardless of the initial cell density of seeding (Figure 6). The amount of particulate ALP released into the medium was directly related to the initial seeding density of the cultures (Figure 6). Cell proliferation for more than 28 days was greatest in lightly seeded cultures (Table 1) and inversely related to the initial seeding density.

Calcification of Native Versus *Culture-Derived Matrix Vesicles*

When the concentration of matrix vesicles was adjusted for ALP activity, the native, media-associated and matrix-associated vesicles all showed a significant calcifying activity (Table 2). As in earlier studies, calcium deposition was dependent on the presence of a phosphate ester in the calcifying medium, in this instance 3 mM AMP. Calcification tests were run on vesicles from 7-day cultures be-

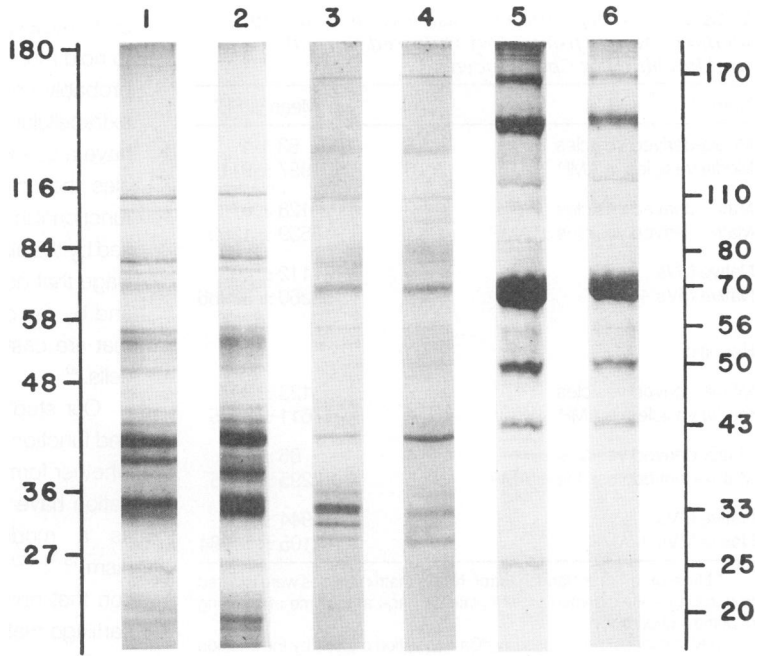

Figure 7. *Major proteins of native and culture-derived matrix vesicles as demonstrated by SDS-polyacrylamide gel electrophoresis. Lane 1 = native vesicles from normal growth plate; Lane 2 = native rachitic vesicles; Lane 3 = cell matrix-derived vesicles from cultured normal chondrocytes, day 7; Lane 4 = cell matrix vesicles from cultured rachitic chondrocytes, day 7; Lane 5 = media-derived vesicles from cultured normal chondrocytes, day 7 culture; Lane 6 = media-derived vesicles from cultured rachitic chondrocytes, day 7 culture. Molecular weights indicated in the left vertical scale are those observed with standards. Molecular weights indicated in the right vertical scale indicate approximate Mr of the major common protein bands.*

cause previous data had shown that this was a period in culture when significant amounts of ALP-positive vesicles could be obtained from both medium and matrix (Figures 1, 2).

Protein Content of Native and Culture-derived Matrix Vesicles

Polyacrylamide gel electrophoresis revealed a similar pattern of major proteins in native, cell matrix-associated and media-associated vesicles whether derived from rachitic or normal chondrocytes (Figure 7). A recurrent pattern of gel bands was observed, with fewer major bands than were seen in isolated chondrocyte plasma membranes.[41] Major bands in native matrix vesicles were seen at approximately 110 kd, a cluster of bands (often a triplet) from 70 to 80 kd, a triplet at 50 to 56 kd, including a strong band at 50 kd, a single or double band at 40 to 43 kd, a doublet at 30 to 35 kd, a single band at 25 kd, a doublet at 17 to 20 kd plus variable fused bands of less than 15 kd molecular weight. Absolute identification has not been made for any of the gel bands. Nevertheless, it is likely that the band at 80 kd represents bone alkaline phosphatase,[42,43] and the strong band(s) at 40 to 45 kd may or may not represent actin because actin has been reported[44,45] but not confirmed[28] in isolated matrix vesicles. It is possible that the strong band seen at approximately 30 kd in native and culture-derived matrix vesicles released by collagenase could represent calpactin II because this 30- to 36-kd calcium-binding protein recently has been reported in chicken matrix vesicles.[46]

Table 1. *Matrix Vesicle Biogenesis in 28-Day Cultures of Rat Epiphyseal Chondrocytes Varying Seeding Density*

Cells/cm^2 day 0 ($\times 10^4$)	Cells/plate day 0 ($\times 10^6$)	Cells/plate day 28 ($\times 10^6$)	Increase in cell count day 28
20.0	2.0	3.1	1.6 ×
5.2	0.5	1.95	3.9 ×
2.6*	0.25	1.62	6.5 ×
1.3	0.125	ND	ND

* Seeding density used by Wuthier et al. (1985).

The protein profiles of vesicles released from matrix by collagenase (Figure 7) are essentially identical, whether from rachitic or normal chondrocytes, and show a close resemblance to native matrix vesicles with major proteins migrating at approximately 110 kd, 70 to 80 kd (triplet), 50 to 55 kd (triplet), 40 to 45 kd, and 30 to 33 kd. There are, however, two unmatched high molecular weight proteins at approximately 170 and 135 kD in cell matrix-derived MVs, and the matrix-derived vesicles lack lower molecular weight species less than 30 kD.

The protein profiles of 7-day media-derived vesicles (Figure 7) are essentially identical whether from rachitic or normal chondrocytes. Although the media-derived vesicles possess several proteins matching those seen in native MVs, at 80 to 85 kd, 70 kd, 50 kd, 43 kd, and 25 kD, there are some nonmatching major proteins at 170 kd, 140 kd, and 116 kD and the band at 65 to 70 kD in media vesicles is very intense, which suggests that absorption or sequestration of albumin has occured. Overall, the gel patterns for media vesicles are not as good a match with native vesicle proteins as are those of the matrix-associated vesicle proteins.

Table 2. *^{45}Ca Deposition by Vesicles Released into Media or Matrix from 7-Day Cultured Normal and Rachitic Rat Chondrocytes*

Normal	N	Mean ± SE*
Media-derived vesicles	3	53 ± 4
Media vesicles + AMP	3	1957 ± 911
Matrix-derived vesicles	2	128 ± 67
Matrix-derived vesicles ± AMP	4	3539 ± 1260
Native MVs	5	112 ± 49
Native MVs + AMP	5	53260 ± 32866
Rachitic		
Media-derived vesicles	2	123 ± 39
Media vesicles + AMP	4	17611 ± 8055
Matrix-derived vesicles	4	86 ± 12
Matrix-derived vesicles + AMP	5	2295 ± 1615
Native MVs	6	944 ± 484
Native MVs + AMP	6	42105 ± 11984

* Mean of CPMs ± standard error. Native matrix vesicles were isolated from collagenase-digested growth plate cartilage at the time of culturing of primary chondrocytes.

In all instances the increase in ^{45}Ca deposition caused by the addition of 3mM AMP was significant at or below the 0.05 confidence interval using the Student's *t*-test.

Discussion

The pattern of *in vitro* matrix vesicle production differs significantly in mammalian *versus* avian chondrocyte cultures. In rat chondrocyte cultures, the vesicles produced are mostly entrapped and sequestered by newly formed cartilage matrix rather than being released into the culture media, as was the case with chicken chondrocyte cultures.[16,26,27] Furthermore, there apparently is no late burst of vesicle production from rat chondrocytes (Figure 6) similar to what was described from chicken chondrocytes.[16,28] It is important for investigators who wish to use mammalian chondrocyte-produced MVs as a model for MV biogenesis and calcification to be aware of these differences.

However, there are many similarities between avian and mammalian MVs produced *in vitro*, including high alkaline phosphatase activity,[16,26,27,29,31,32] ^{45}Ca-depositing activity,[16,26,27] and lipid and phospholipid content.[16,26,31] Furthermore, the studies reported here have shown that culture-derived mammalian MVs resemble their *in vivo* counterpart (native MVs) closely in having high-alkaline phosphatase activity, calcifiability as tested by the centrifugal method,[13,33,37] and the presence of a characteristic, simplified protein profile on SDS-PAGE that demonstrates similar major protein species.[16,41,44,47] Thus culture-derived MVs, either from rat or chicken chondrocytes, appear to represent a good test model for studies of MV biochemical composition and MV-initiated calcification. Examination of the mechanism of *in vitro* MV biogenesis and physiologic regulators of MV biogenesis would seem to hold much promise as an approach to analysis of this, probably universal, mechanism by which cells generate extracellular vesicles. Extracellular vesicles either may have a biologic purpose, as is the case with matrix vesicles that initiate calcification,[2,13,14] or they may not be functional in the host's metabolism, as would be exemplified by the alkaline phosphatase-poor MVs of articular cartilage that do not normally calcify[48,49] (except in arthritis), and by the circulating, enzyme-active vesicular particles that are cast into the blood stream by malignant tumor cells.[50]

Our studies also document the similarity in structure and function between normal and rachitic matrix vesicles, whether formed *in vivo* or *in vitro*. Many studies of calcification have used rachitic growth plate and metaphysis as a model for the normal calcification mechanism.[15,33-35,51-53] This rationale is based on the assumption that normal and rachitic cells, MVs, and bone and cartilage matrix are basically similar, and on the fact that rachitic tissues are more conveniently obtained in a relatively uncalcified condition so that the entire calcification cascade awaits the addition of key factors and conditions to be activated. Our demonstration that normal native and culture-derived MVs resemble comparable rachitic MVs in having high ALP content, an almost identical content of major proteins, and ready calcifiability *in vitro* strengthens the assumption that the mechanism of calcification in rachitic bones and MVs is essentially normal and, as such, represents a valid model for normal calcification.

Finally, it is apparent that rat chondrocyte cultures gradually dedifferentiate during prolonged cultivation. Evidence presented above for this conclusion includes 1) the steadily decreasing output of ALP-enriched vesicles into both medium and newly formed matrix after 1 to 2 weeks in primary culture; 2) the fact that media vesicle production does not resume between 2 and 4 weeks of continuous primary cultivation; 3) the progressive decline in specific activity of ALP in cells during 4 weeks in culture; and 4) the fact that less dense seeding of chondrocytes is associated with an increased cell proliferation and decreased expression of ALP activity. Thus the rapidity of loss of chondrocyte phenotypic expression appears to be directly related to the amount of cell division occurring during prolonged cultivation, with cultures initially seeded at high density experiencing less cell division and showing greater persistence of ALP activity. This inverse relation between degree of phenotypic expression and the number of cell divisions occurring *in vitro* would seem to argue in favor of using high-density primary cultures of mammalian chondrocytes to study *in vitro* MV biogenesis and calcification, rather than using higher-passage cultures and/or primary cultures that were seeded at a low density.

References

1. Akisaka T, Gay CV: The plasma membrane and matrix vesicles of mouse growth plate chondrocytes during differentiation as revealed in freeze-fracture replicas. Am J Anat 1985, 173:269–286
2. Anderson HC: Vesicles associated with calcification in the matrix of epiphyseal cartilage. J Cell Biol 1969, 41:59–72
3. Anderson HC, Johnson TF, Avramides A: Matrix vesicles in osteomalacic bone. Metab Bone Dis Rel Res 1980, 2S:79–86
4. Bernard GW, Pease DC: An electron microscopic study of initial intramembranous osteogenesis. Am J Anat 1969, 125: 271–290
5. Bernard GW: Ultrastructural observations of initial calcification in dentine and enamel. J Ultrastr Res 1972, 41:1–17
6. Bonucci E: Fine structure and histochemistry of calcifying globules in epiphyseal cartilage. Z Zellforsch Mikr Anat 1970, 103:192–217
7. Cecil RNA, Anderson HC: Freeze-fracture studies of matrix vesicle calcification in epiphyseal growth plate. Metab Bone Dis Rel Res 1978, 1:89–97
8. Eisenman DR, Glick PL: Ultrastructure of initial crystal formation in dentin. J Ultrastr Res 1972, 41:18–28
9. Martino LJ, Yaeger VL, Taylor JJ: An ultrastructural study of the role of calcification nodules in the mineralization of woven bone. Calcif Tiss Int 1979, 27:57–64
10. Ozawa H, Najima T: Ultrastructure and cytochemistry of matrix vesicles in developing cartilage and tooth germ. Fourth International Congress on Histochemistry and Cytochemistry, 1972, Kyoto, pp. 311–312
11. Sisca RF, Provenza DV: Initial dentin formation in human deciduous teeth. An electron microscopic study. Calc Tiss Res 1972, 9:1–16
12. Slavkin HC, Bringas P Jr, Croissant R, Bavetta LA: Epithelial-mesenchymal interactions during odontogenesis II. Intercellular matrix vesicles. Mech Age Dev 1972, 1:1–23
13. Ali SY, Evans L: The uptake of (^{45}Ca) calcium Ions by matrix vesicles isolated from calcifying cartilage. Biochem J 1973, 134:647–650
14. Anderson HC, Reynolds JJ: Pyrophosphate stimulation of calcium uptake into cultured embryonic bones. Fine structure of matrix vesicles and their role in calcification. Develop Biol 1973, 34:211–227
15. Anderson HC, Hsu HHT: A new method to measure ^{45}Ca accumulation by matrix vesicles in slices of rachitic growth plate cartilage. Metab Bone Dis Rel Res 1978, 1:193–198
16. Wuthier RE, Chin JE, Hale JE, Register TC, Hale LV, Ishikawa Y: Isolation and characterization of calcium-accumulating matrix vesicles from chondrocytes of chicken eipihyseal growth plate cartilage in primary culture. J Biol Chem 1985, 260:15972–15979
17. Cotmore JM, Nichols G Jr, Wuthier RE: Phospholipid-calcium phosphate complex: Enhanced calcium migration in the presence of phosphate. Science 1971, 172:1339–1341
18. Peress NS, Anderson HC, Sajdera SW: The lipids of matrix vesicles from bovine fetal epiphyseal cartilage. Calcif Tiss Res 1974, 14:275–281
19. Wuthier RE. Lipid composition of isolated cartilage cells, membranes and matrix vesicles. Biochim Biophys Acta 1975, 409:128–143
20. Wuthier RE, Gore ST: Partition of inorganic ions and phospholipids in isolated cell, membrane and matrix vesicle fractions: Evidence for Ca-Pi-acidic phospholipid complexes. Calcif Tiss Res 1977, 24:163–171
21. Rabinovitch AL, Anderson HC: Biogenesis of matrix vesicles in cartilage growth plates. Fed Proc 1976, 35:112–116
22. Kardos TB, Hubbard MJ: Are matrix vesicles apoptotic bodies? Factors and Mechanisms Influencing Bone Growth. New York, Alan R. Liss, 1982, pp. 45–60
23. Akisaka T, Shigenaga T: Ultrastructure of growing epiphyseal cartilage processed by rapid freezing and freeze substitution. J Electron Microsc 1983, 32:305–320
24. Rabinovitch AL: Autoradiographic studies of lipid synthesis and transport in growth plate cartilage of mice. Fed Proc 1974, 33:617
25. Akisaka T, Kawaguchi H, Subita GP, Shigenaga Y, Gay CV: Ultrastructure of matrix vesicles in chick growth plate as revealed by quick freezing and freeze substitution. Calcif Tiss Int 1988, 42:383–393
26. Glaser JH, Conrad HE: Formation of matrix vesicles by cultured chick embryo chondrocytes. J Biol Chem 1981, 256: 2607–2611
27. Golub EE, Schattschneider SC, Berthold P, Burke A, Shapiro IM: Induction of chondrocyte vesiculation *in vitro*. J Biol Chem 1983, 258:616–621
28. Hale JE, Wuthier RE: The mechanism of matrix vesicle formation. Studies on the composition of chondrocyte microvilli and on the effects of microfilament-perturbing agents on cellular vesiculation. J Biol Chem 1987, 262:1916–1925
29. Anderson HC, Collins DE, Jacobs DH, Morris DC, Hsu HHT: Matrix vesicle biogenesis and release in micromass cultures of rachitic rat chondrocytes. Bone 1985, 6:477
30. Vaananen HK, Morris DC, Anderson HC: Calcification of cartilage matrix in chondrocyte cultures derived from rachitic growth plate. Metal Bone Dis Rel Res 1983, 5:87–93
31. Boyan B, Schwartz Z, Swain L, Carnes D, Zislis T: Differential expression of phenotype by resting and growth region costochondral chondrocytes *in vitro*. Bone 1988, 9:185–194
32. Schwartz Z, Knight G, Boyan B: Localization of vitamin D responsive alkaline phosphatase in costochondral chondrocyte cultures. J Biol Chem 1988, 263:6023–6026
33. Murphree S, Hsu HHT, Anderson HC: *In vitro* formation of crystalline apatite by matrix vesicles isolated from rachitic rat epiphyseal cartilage. Calcif Tiss Int 1982, 34:S62–S68
34. McCollum EB, Simmons N, Shipley PG, Park EA: Studies on experimental rickets: A delicate biological test for calcium depositing substances. J Biol Chem 1922, 51:41–49
35. Anderson HC, Cecil R, Sajdera SW: Calcification of rachitic rat cartilage *in vitro* by extracellular matrix vesicles. Am J Path 1975, 79:237–255
36. Ali SY, Sajdera SW, Anderson HC: Isolation and characterization of calcifying matrix vesicles from epiphyseal cartilage. Proc Nat Acad Sci (USA) 1970, 67:1513–1520
37. Hsu HHT, Anderson HC: A simple and defined method to study calcification by isolated matrix vesicles. Effect of ATP

and vesicle phosphatase. Biochim Biophys Acta 1977, 500: 162–172
38. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein management with the folin phenol reagent. J Biol Chem 1951, 193:265–275
39. Matsuzawa T, Anderson HC: Phosphatases of epiphyseal cartilage studied by electron microscopic cytochemical methods. J Histochem Cytochem 1971, 19:801–808
40. Laemmli VK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970, 227: 680–685
41. Bohn WW, Stein RM, Hsu HHT, Morris DC, Anderson HC: Isolation of a plasma membrane-enriched fraction from collagenase suspended rachitic rat growth plate chondrocytes. J Orthop Res 1984, 1:319–324
42. Hsu HHT, Munoz PA, Barr J, Oppliger I, Morris DC, Vaananen HK, Tarkenton N, Anderson HC: Purification and partial characterization of alkaline phosphatase of matrix vesicles from bovine epiphyseal cartilage. Purification by monoclonal antibody affinity chromatography. J Biol Chem 1985, 260: 1826–1831
43. Nair BC, Johnson DE, Majeska RJ, Rodkey JA, Bennett CD, Rodan GA: Rat alkaline phosphatase. II structural similarities between osteosarcoma, bone, kidney and placental isoenzymes. Arch. Biochem. Biophys. 1987, 254:28–34
44. Muhlrad A, Stein H, Bab IA, Sela J: Fine structure and enzymes of matrix vesicles in osteosarcoma: Possible occurrence of contractile proteins. Metab Bone Dis Rel Res 1978, 1:227–233
45. Sela J, Bab IA, Muhlrad A: Ultrastructural and biochemical characterization of extracellular matrix vesicles in healing alveolar bone sockets: preliminary indications for the presence of contractile proteins. Metab Bone Dis Rel Res 1978, 1:185–192
46. Genge BR, Wu LNY, Wuthier RE: Identification of phospholipid-dependent calcium binding proteins as constituents of matrix vesicles. J Biol Chem 1989, 264:10917–10921
47. Stein RM, Hsu HHT, Anderson HC: Protein profiles of isolated fetal calf and rachitic rat matrix vesicles by polyacryalmids gel electrophoresis. Proceedings of the Third International Conference on Matrix Vesicles, 1981. Edited by Ascenzi A, Bonucci E, DeBernard B. Milano, Wichtig Editore, pp. 117–122
48. Ali SY, Bayliss MT: Enzymic changes in human osteoarthritic cartilage. Normal and Osteoarthritic Cartilage. London, Institute of Orthopaedics Publication, 1984, pp. 189–205
49. Ali SY: Matrix vesicles and apatite nodules in arthritic cartilage. Perspectives in Inflammation. Future Trends and Developments. Lancaster, England, MTP Press, 1977, pp. 211–223
50. Losa GA, Heumann D, Carrel S, Von Fliedner V, Mach JP: Characterization of membrane vesicles circulating in the serum of patients with common acute lymphoblastic leukemia. Lab Invest 1986, 56:573–579
51. Anderson HC, Sajdera SW: Calcification of rachitic cartilage to study matrix vesicle function. Fed Proc 1976, 35:148–153
52. Fallon MD, Whyte MP, Teitlebaum SL: Stereospecific inhibition of alkaline-phosphatase by L-tetramisole prevents *in vitro* cartilage calcification. Lab Invest 1980, 43:489–494
53. Johnson TF, Morris DC, Anderson HC: Matrix vesicles and calcification of rachitic rat osteoid. J Exp Path 1989, 4:123–132