Cells and Materials

Volume 2 | Number 2 Article 6

1992

Extracellular Matrix Vesicles in Endochondral Bone Development and in Healing After Injury

J. Sela
The Hebrew University Hadassah Faculty of Dental Medicine

Z. Schwartz
The Hebrew University Hadassah Faculty of Dental Medicine

L. D. Swain
The University of Texas Health Science Center

D. Amir
The Hebrew University Hadassah Faculty of Dental Medicine

B. D. Boyan
The University of Texas Health Science Center

CELLS AND MATERIALS, Vol. 2, No. 2, 1992 (pages 153-161) 1051-6794/92$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

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

J. Sela[1]*, Z. Schwartz[2], L.D. Swain[4], D. Amir[3], and B.D. Boyan[4]

[1]Division of Oral Pathology and the Laboratory of Biomineralization,
[2]Department of Periodontics, and [3]Orthopaedic Surgery,
The Hebrew University Hadassah Faculty of Dental Medicine, Jerusalem, Israel 91-010,
[4]Department of Orthopaedics, The University of Texas Health Science Center, San Antonio, Texas 78284

(Received for publication December 9, 1991, and in revised form March 2, 1992)

Abstract

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

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

*Address for Correspondence:
Jona Sela
Division of Oral Pathology
and Laboratory of Biomineralization
Hebrew University
Hadassah Faculty of Dental Medicine
P.O. Box 1172
Jerusalem 91010, Israel
Phone No.:(972) 2-428576

Introduction

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

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

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

Although isolated matrix vesicles support hydroxyapatite formation *in vitro* [9, 72], a critical question in the biogenesis of matrix vesicles has been whether or not

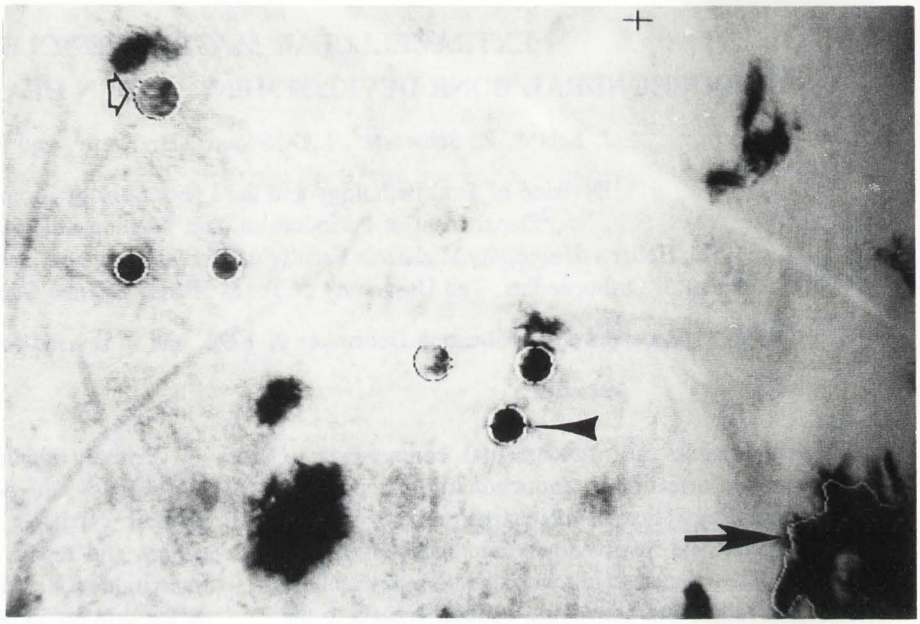

Figure 1: Transmission electron micrograph photographed from television monitor to demonstrate the method for morphometric quantitation of extracellular matrix vesicles in healing bone. Typing and measurement of diameter and distance from the calcified front is performed by vesicular detection with a pointing device (+) to encircle their outline and delineation of the calcified front (arrow). Transopaque matrix vesicle is indicated by an arrowhead and transparent matrix vesicle by an open arrowhead. Photo width = 5.7 μm.

they are "preloaded" with calcium and phosphate at the time of their release. Specific interactions of Ca^{2+} and phosphate ions with phospholipids in the cell membrane could result in the formation of CPLX [28]. Phospholipid-mineral interactions may be responsible for membrane fusion, allowing vesicular budding into the extracellular matrix [37, 72]. The occurrence of actin in isolated vesicle fractions is indicative of a cytoskeletal role in vesicle formation as well [45, 61, 72].

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

Changes in Matrix Vesicle Morphology and Biochemistry in Bone Healing

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

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

Changes in matrix vesicle morphometry correlate with biochemical changes in matrix vesicle-enriched microsomal fractions (MVEM) isolated from the healing tissue. At six days post-injury, there is an increase in MVEM alkaline phosphatase and phospholipase A_2 specific activities (Fig. 2) [57]. Phospholipid composition changes with time. The amount of phosphatidylcholine decreases as a function of time. Phosphatidylserine content is greatest at days 6 and 14, which correlates with

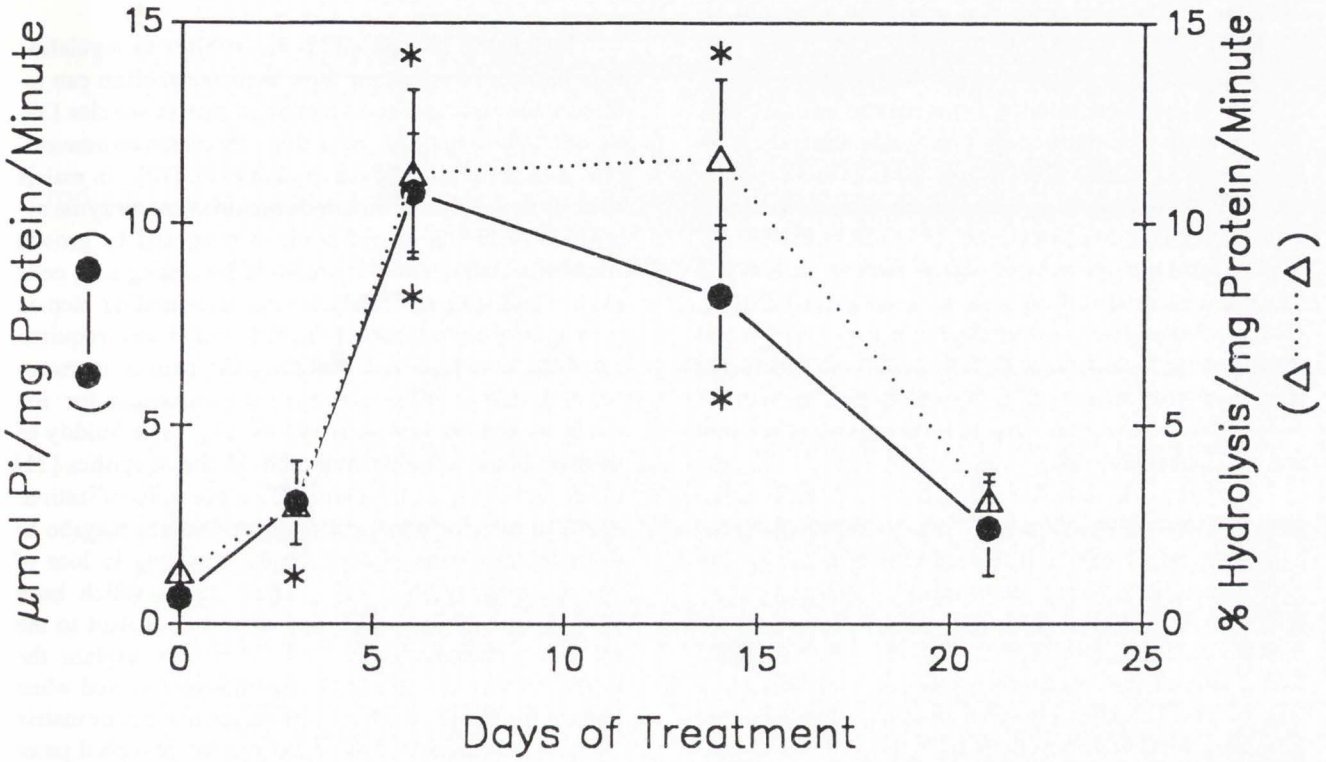

Figure 2: Specific activities of alkaline phosphatase expressed as μmol P_i/mg protein/minute and phospholipase A_2 expressed as percent hydrolysis/mg protein/minute in matrix vesicle-enriched membranes isolated from tibial bone healing following marrow ablation. Isolation is performed by differential centrifugation of homogenates of the newly formed tissues. Values are presented as means ± standard error of mean (S.E.M.) for six samples, each sample composed of pooled tissue of six animals. *: statistically significant; $p < 0.05$ for each time point compared to time 0.

---

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

Matrix vesicle morphometrics and MVEM biochemical parameters are sensitive to regulation *in vivo*. When bone-bonding and non-bonding ceramic and metal implants were inserted into the marrow-ablated tibia, changes were observed in the normal healing pattern, both in the treated leg and in MVEM isolated from the contralateral limb [7, 44, 53]. Bone-bonding materials stimulated MVEM alkaline phosphatase specific activity and increased matrix vesicle density in the treated limb at early time points. While MVEM alkaline phosphatase behaved like that seen in normal healing at later times, matrix vesicle concentration (#/μm^2) was decreased with respect to normal. MVEM phospholipase A_2 specific activity was lower in tissue adjacent to the bone-bonding implants and was markedly inhibited in the contralateral limb.

The implant-dependent decrease in MVEM phospholipase A_2 activity was even more marked in the tissue adjacent to nonbonding implants; there was no MVEM phospholipase A_2 activity in the contralateral limb. MVEM alkaline phosphatase specific activity became only slightly elevated during healing, but no differences in magnitude between treatment and control limbs were evident. Matrix vesicle density was either comparable to that of normal healing bone or was depressed. These data demonstrate that there is both local and systemic regulation involved.

Role of Matrix Vesicle Components in Mineral Deposition

The data presented above show that changes in alkaline phosphatase or phospholipase A_2 activities can be correlated with altered matrix vesicle function *in vivo*. Other components of the vesicle are now known to play a role in the calcification process. Most information is

derived from studies of cartilage and cartilage cells. Initial sites of hydroxyapatite deposition are observed on the inner vesicle membranes [1-3, 9]. CPLX and proteolipids, shown to support calcification *in vitro* [16, 17, 23, 50], have been isolated from matrix vesicles [23, 73], suggesting that they could function as mineralization initiation sites. Other observations support this hypothesis. CPLX is present at sites where mineralization is about to occur or has just started, as evidenced in hypertrophic cartilage, metaphyseal bone, fracture callus, and following induction of heterotopic bone formation [10, 16-20]. Proteolipids are enriched in matrix vesicles and participate in formation of CPLX, in part by structuring phosphatidylserine [21, 22]. Proteolipids may also act as ionophores, exporting protons and importing calcium and phosphate [27, 68].

Enzymes like alkaline phosphatase, pyrophosphatase, ATPase, phospholipase A_2, and NTP-pyrophosphohydrolase, which exhibit increased activity as the growth plate mineralizes, have been localized to matrix vesicles [32, 33, 38, 70] and are regulated by factors which monitor calcification [24, 55, 56, 58]. Robison [52] first observed the relationship between bone formation and increased alkaline phosphatase and postulated a role in raising local concentrations of P_i [9, 40]. It is possible that more than one matrix vesicle alkaline phosphatase exists [11], each with a distinct function.

Other matrix vesicle proteins appear to play a role in calcification. Uptake of mineral ions by isolated matrix vesicles incubated in synthetic cartilage lymph has been shown to follow a consistent pattern. After an initial lag, vesicles rapidly accumulated Ca^{2+} and P_i, an ability readily destroyed by proteases. A subset of EGTA-extractable proteins selectively bound Ca^{2+} in the presence of lipids and cross-reacted with antibodies to calpactin II, suggesting that this protein was involved in ion uptake [34].

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

Regulation of Matrix Vesicle Mineral Formation

Membrane phospholipids are subject to regulation by a number of enzymes; thus, their metabolism can influence the structure and function of matrix vesicles [39, 64-66]. Phospholipase A_2 activity increases commensurate with maturation of the growth plate [70]. In matrix vesicles isolated from cultured chondrocytes, enzyme activity is higher in growth vesicles produced by growth zone cells than in vesicles produced by resting zone cells [55]. This enzyme catalyzes the rate-limiting step in prostaglandin production [13, 36], and is also required for fatty acid turnover, resulting in changes in membrane fluidity. This can have consequences for ion transport and enzyme activity [46, 51]. The fluidity of the membrane can alter its structural characteristics [31] ultimately changing the ability to support mineralization. Another role for phospholipases in vesicles may be to degrade membrane phospholipids, resulting in loss of vesicle integrity [67, 72]. Those lipids which have formed mineral complexes appear to be resistant to the action of phospholipases [50]. This may explain the difference in susceptibility to hydrolysis observed when phospholipids isolated from plasma membranes or matrix vesicles are incubated with exogenous phospholipases [58].

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

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

References

1. Ali SY (1976) Analysis of matrix vesicles and their role in the calcification of epiphyseal cartilage. Fed Proc Am Soc Exp Biol 35, 135-142.

2. Ali SY (1980) Mechanism of calcification. In: Scientific Foundations of Orthopaedics and Traumatology, Owen R, Goodfellow J, Bollough P (eds.), Heinemann, London, pp 175-195.

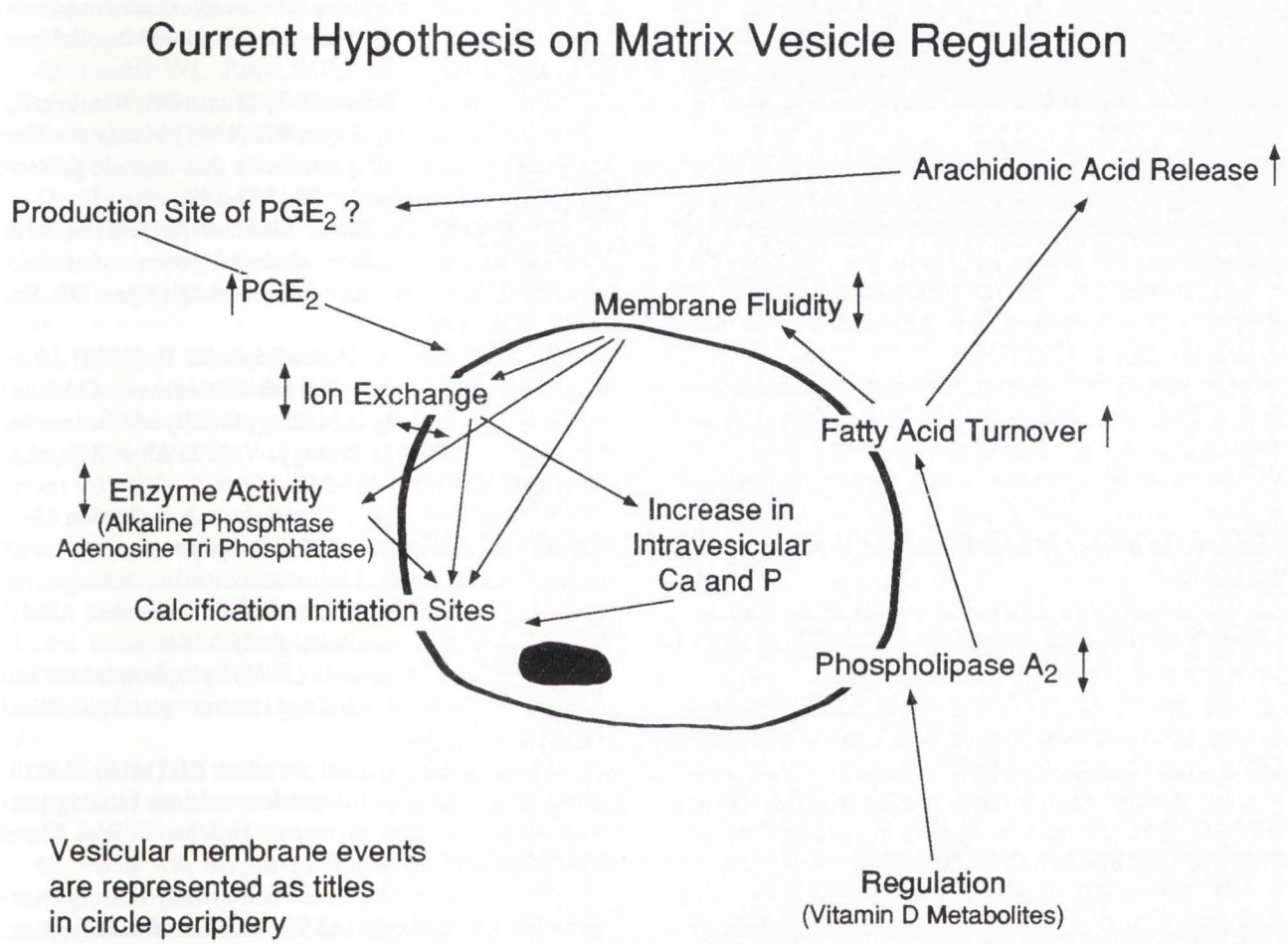

Figure 3. Current hypothesis on matrix vesicle regulation. This diagram demonstrates a possible chain of events and their suggested localization regulating the process of initial calcification within extracellular matrix vesicles, the data are taken from literature cited in the text.

3. Ali SY (1983) Calcification of cartilage. In: Cartilage, Structure Function and Biochemistry, Vol. 1, Hall BK (ed.), Academic Press, pp 343-379.

4. Ali SY, Evans L (1973) The uptake of ^{45}Ca ions by matrix vesicles isolated from calcifying cartilage. Biochem J **134**, 647-650.

5. Amir D, Schwartz Z, Sela J, Weinberg H (1988) The relationship between extracellular matrix vesicles and calcifying front on the 21st day after injury to rat tibial bone, Clin Orthop Rel Res **230**, 289-295.

6. Amir D, Schwartz Z, Weinberg H, Sela J (1988) The distribution of extracellular matrix vesicles in healing tibial bone 3 days after intermedullary injury. Arch Orthop Trauma Surg **107**, 1-6.

7. Amir D, Sela J, Müller-Mai C, Wendland H, Gross S (1989) Effects of glass-ceramic implants on primary calcification in rat tibial bone after injury. Biomaterials **10**, 585-589.

8. Anderson HC (1976) Matrix vesicles of cartilage and bone. In: The Biochemistry and Physiology of Bone, Vol. 14, Bourne GH (ed.), Academic Press, pp 135-151.

9. Anderson HC (1985) Matrix vesicle calcification: Review and update. In: *Bone and Mineral Research*, Vol. 3, Pick WA (ed.), Excerpta Medica, Amsterdam, pp 109-149.

10. Anghileri LJ (1972) Phospholipid-calcium complexes in experimental tumors. Experientia **28**, 1086-1087.

11. Arsenis C, Hackett MH, Huang SM (1976) Resolution specificity and transphosphorylase activity of calcifying cartilage alkaline phosphatases. Calcif Tissue Res **20**, 159-171.

12. Bab I, Schwartz Z, Deutsch D, Muhlrad A, Sela J (1983) Correlative morphometric and biochemical analysis of purified extracellular matrix vesicles from rat

alveolar bone. Calcif Tissue Int **35**, 320-326.

13. Bell RL, Kennerly DA, Standford N, Majerus PW (1979) Diglyceride lipase: a pathway for arachidonate release from human platelets. Proc Natl Acad Sci USA, **76**, 3238-3241.

14. Barckhaus, RH, Krefting ER, Althoff R, Quint P, Höhling HJ (1981) Electron microscopic microprobe analysis on the initial stages of mineral formation in the epiphyseal growth plate. Cell Tissue Res **217**, 661-666.

15. Boskey AL (1981) Current concepts of the physiology and biochemistry of calcification. Clin Orthop Rel Res **157**, 225-257.

16. Boskey AL, Posner AS (1976) Extraction of a calcium phospholipid-phosphate complex from bone. Calcif Tissue Int **19**, 273-283.

17. Boskey AL, Posner AS (1977) The role of synthetic and bone-extracted Ca-phospholipid-PO_4 complexes in hydroxyapatite formation. Calcif Tissue Int **23**, 251-258.

18. Boskey AL, Posner AS (1977) *In vitro* nucleation of hydroxyapatite by a bone calcium phospholipid phosphate complex. Calcif Tissue Int **22**, 197-201.

19. Boskey AL, Reddi AH (1983) Changes in lipids during matrix-induced-endochondral bone formation. Calcif Tissue Int **35**, 549-554.

20. Boskey AL, Timchak DM, Lane JM, Posner AS (1980) Phospholipid changes during fracture healing. Proc Soc Exp Biol Med **165**, 368-373.

21. Boyan BD, Boskey AL (1984) Co-isolation of proteolipids and calcium-phospholipid-phosphate complexes, Calcif Tissue Int **36**, 214-218.

22. Boyan-Salyers BD, Boskey AL (1980) Relationship between proteolipids and calcium-phospholipid-phosphate complexes in *Bacterionema matruchotii* calcification. Calcif Tissue Int **30**, 167-174.

23. Boyan-Salyers BD, Vogel JJ, Riggan LJ, Summers F, Howell RE (1978) Application of a microbial model to biologic calcification, Metab Bone Dis Rel Res **1**, 143-147.

24. Boyan B, Schwartz Z, Carnes D, Ramirez V (1988) The effects of vitamin D metabolites on the plasma and matrix vesicle membranes of growth and resting cartilage *in vitro*, Endocrinology **122**, 2851-2860.

25. Boyan BD, Schwartz Z, Swain LD (1992) *In vitro* studies on the regulation of endochondral ossification by vitamin D. Crit Rev Oral Biol Med **3**, 15-30.

26. Buckwalter JA, Ehrlich MG, Armstrong AL, Mankin HJ (1987) Electron microscopic analysis of articular cartilage proteoglycan degradation by growth-plate enzyme, J Orthop Res **5**, 128-132.

27. Campbell JW, Speeg KV (1969) Ammonia and the biological deposition of calcium carbonate. Nature **224**, 725-726.

28. Cotmore JM, Nichols GJ, Wuthier RE (1971) Phospholipid calcium phosphate complex: enhanced calcium migration in the presence of phosphate. Science **172**, 1339-1341.

29. Dean DD, Schwartz Z, Muniz OE, Ramirez V, Swain L, Howell DS, Boyan BD (1991) Matrix vesicles are enriched in metalloproteinases that degrade proteoglycans. Calcif Tissue Int **50**, 342-349.

30. Deutsch D, Bab I, Muhlrad A, Sela J (1982) Purification and further characterization of matrix vesicles from rat alveolar bones. Metab Bone Dis Rel Res **3**, 209-214.

31. Duzgunes N, Paphadjopoulas D (1983) Ionotropic effects on phospholipid membranes: Calcium/magnesium specificity in binding, fluidity and fusion. In: Membrane Fluidity in Biology, Vol. 2, Aloia RC (ed.), Academic Press, pp 187-216.

32. Einhorn TA, Hirschman A, Gordon SL, Avitable MJ, Carty RP (1986) Enzymes associated with matrix vesicles isolated from osteoarthritic cartilage. In: Cell Mediated Calcification and Matrix Vesicles, Ali SY (ed.), Elsevier, Amsterdam, pp 359-364.

33. Felix R, Fleisch H (1976) Pyrophosphatase and ATPase of isolated cartilage matrix vesicles. Calcif Tissue Res **22**, 1-7.

34. Gene BR, Wu LN, Wuthier RE (1989) Identification of phospholipid-dependent calcium binding proteins as constituents of matrix vesicles. J Biol Chem **264**, 10917-10921.

35. Glimcher MJ, Krane SM (1968) The organization structure of bone and mechanism of calcification. In: *Treatise on Collagen*, Vol. IIB, Ramachandran GN, Gould BS (eds.), Academic Press, pp 68-251.

36. Hakeda Y, Yoshino T, Natakani Y, Kurihara N, Maeda N, Kumegawa M (1986) Prostaglandin E_2 stimulates DNA synthesis by a cyclic AMP independent pathway in osteoblastic clone MC35C-E1 cells. J Cell Physiol **128**, 155-161.

37. Hale JE, Wuthier RE (1987) 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 **262**, 1916-1925.

38. Hirschman A, Deutsch D, Hirschman M, Bab I, Sela J, Muhlrad A (1983) Neutral peptidase activities in matrix vesicles from bovine fetal alveolar bone and dog osteosarcoma. Calcif Tissue Int **35**, 791-797.

39. Irving JT, Wuthier RE (1968) Histochemistry and biochemistry of calcification with special reference to the role of lipids. Clin Orthop **56**, 236-260.

40. Kahn SE, Jafri AM, Lewis NJ, Arsenis C (1978) Purification of alkaline phosphatase from extracellular matrix vesicles of fracture callus cartilage. Calcif Tissue Res **25**, 85-92.

41. Kawabe N, Ehrlich MG, Mankin HJ (1986) *In*

vivo degradation systems of the epiphyseal cartilage. Clin Orthop **211**, 244-251.

42. Landis WJ, Paine MC, Glimcher MJ (1977) Electron microscopic observations of bone tissue prepared anhydrously in organic solvents. J Ultrastruct Res **59**, 1-30.

43. Majeska RJ, Wuthier RE (1975) Studies on matrix vesicles isolated from chick epiphyseal cartilage: association of pyrophosphatase and ATPase activities with alkaline phosphatase. Biochim Biophys Acta, **391**, 51-60.

44. Marshall TS, Schwartz Z, Swain LD, Amir D, Sela J, Gross U, Müller-Mai C, Boyan BD (1991) Matrix vesicle enzyme activity in endosteal bone following implantation of bonding and nonbonding implant materials. Clin Oral Implants Res **2**, 112-120.

45. Muhlrad A, Bab IA, Deutsch D, Sela J (1982) The occurrence of actin-like protein in extracellular matrix vesicles. Calcif Tissue Int **34**, 376-381.

46. Nemere I, Yoshimoto Y, Norman AW (1984) Calcium transport in perfused duodena from normal chicks enhancement within fourteen minutes of exposure to 1.25 dihydroxyvitamin D_3, Endocrinology **115**, 1476-1483.

47. Peress NS, Anderson HC, Sajdera SW (1974) The lipids of matrix vesicles from bovine fetal epiphyseal cartilage. Calcif Tissue Int **14**, 275-281.

48. Poole AR, Matsui Y, Hinek A, Lee ER (1989) Cartilage macromolecules and the calcification of cartilage matrix. Anat Rec **224**, 167-179.

49. Poole AR, Pidoux I, Rosenberg L (1982) Role of proteoglycans in endochondral ossification: immunofluorescent localization of link protein and proteoglycan monomer in bovine fetal epiphyseal growth plate. J Cell Biol **92**, 249-260.

50. Raggio CL, Boyan BD, Boskey AL (1986) *In vivo* hydroxyapatite formation induced by lipids, J Bone Min Res **1**, 409-415.

51. Rasmussen H, Matsumoto T, Fontaine O, Goodman DB (1982) Role of changes in membrane lipid structure in the action of 1,25 dihydroxyvitamin D_3. Fed Proc **41**, 72-77.

52. Robison R (1923) The possible significance of hexose phosphoric esters in ossification. Biochemistry **17**, 286-293.

53. Schwartz Z, Amir D, Boyan BD, Kohavi D, Müller-Mai C, Swain LD, Gross U, Sela J (1991) Effect of glass ceramic and titanium implants on primary calcification during rat tibial bone healing. Calcif Tissue Int **49**, 359-364.

54. Schwartz Z, Amir D, Weinberg H, Sela J (1987) Extracellular matrix vesicle distribution in primary mineralization 2 weeks after injury to rat tibial bone. Eur J Cell Biol **45**, 97-101.

55. Schwartz Z, Boyan B (1988) The effects of vitamin D metabolites on phospholipase A_2 activity of growth zone and resting zone cartilage cells *in vitro*. Endocrinology **122**, 2191-2198.

56. Schwartz Z, Knight G, Swain L, Boyan B (1988) Localization of vitamin D-responsive alkaline phosphatase in cultured chondrocytes. J Biol Chem **263**, 6023-6026.

57. Schwartz Z, Sela J, Ramirez V, Amir D, Boyan BD (1989) Changes in extracellular matrix vesicles during healing of rat tibial bone: a morphometric and biochemical study. Bone **10**, 53-60.

58. Schwartz Z, Swain LD, Ramirez V, Boyan BD (1990) Regulation of fatty acid turnover by 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in matrix vesicles and plasma membranes isolated from growth zone and resting zone chondrocyte cultures. Biochim Biophys Acta **102**, 278-286.

59. Sela J, Amir D, Schwartz Z, Weinberg H (1987) Changes in the distribution of extracellular matrix vesicles during healing of rat tibial bone. Bone **8**, 245-250.

60. Sela J, Amir D, Schwartz Z, Weinberg H (1987) Ultrastructural tissue morphometry of the distribution of extracellular matrix vesicles in remodeling rat tibial bone, six days after injury. Acta Anat **128**, 295-300.

61. Sela J, Bab IA, Muhlrad A (1978) 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 **1**, 185-191.

62. Sela J, Brandes J, Strunz I, Bab I, Gross U (1981) Primary mineralization and extracellular matrix vesicles in rat bone after administration of glassceramic implants, Arch Orthop Traumat Surg **98**, 237-243.

63. Sela J, Schwartz Z, Amir D, Rachamim E, Weinberg H (1987) A comparative study on the distribution of extracellular matrix vesicles in rat tibial bone 1 and 3 weeks after injury. In: Current Advances in Skeletogenesis, Vol. 3, Hurwitz S, Sela J (eds.) Heiliger Publ Co, Jerusalem, pp 149-162.

64. Shapiro I (1970) The association of phospholipids with an organic bone. Calcif Tissue Int **5**, 13-20.

65. Shapiro I (1970) The phospholipids of mineralized tissues, I. Mammalian compact bone. Calcif Tissue Int **5**, 21-29.

66. Shapiro IM, Wuthier RE, Irving JT (1966) Phospholipids of bovine dental tissue. I. Enamel matrix and dentine. Arch Oral Biol **11**, 501-512.

67. Simmons DJ (1976) Comparative physiology of bone. In: The Biochemistry and Physiology of Bone, Vol. 4, Bourne GH (ed.), Academic Press, pp 446-516.

68. Swain LD, Boyan BD (1988) Ion-translocating

properties of calcifiable proteolipids. J Dent Res **67**, 526-530.

69. Takagi M, Parmley RT, Denys FR (1981) Endocytic activity and ultrastructural cytochemistry of lysosome related organelles in epiphyseal chondrocytes. J Ultrastruc Res **74**, 69-82.

70. Wuthier RE (1973) The role of phospholipids in biological calcification: distribution of phospholipase activity on calcifying epiphyseal cartilage. Clin Orthop Rel Res **90**, 191-200.

71. Wuthier RE (1975) Lipid composition of isolated epiphyseal cartilage cell membranes and matrix vesicles. Biochim Biophys Acta **409**, 128-143.

72. Wuthier RE (1982) A review of primary mechanism of endochondral calcification with special emphasis on the role of cells, mitochondria and matrix vesicles. Clin Orthop Rel Res **169**, 219-242.

73. Wuthier RE, Gore ST (1977) Partition of inorganic ions and phospholipids in isolated cell, membrane and matrix vesicle fractions, evidence for Ca-P_i-acidic phospholipid complexes. Calcif Tissue Int **24**, 163-171.

74. Wuthier RE, Linder RE, Warner GP, Gore ST, Borg TK (1978) Non-enzymatic isolation of matrix vesicles: characterization and initial studies on ^{45}Ca and ^{32}P-orthophosphate metabolism, Metab Bone Dis Rel Res **1**, 125-136.

Discussion with Reviewers

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

Authors: Recent studies have shown that matrix vesicles are under autocrine regulation by chondrocytes. These cells produce vitamin D metabolites which have direct, nongenomic effects on the matrix vesicle membrane. In effect, the cell is able to regulate events in the matrix by remote control. While 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ have comparable effects on isolated matrix vesicles and plasma membranes, the effects are seen only in the matrix vesicles and not in the plasma membranes isolated from intact cells incubated with hormone. These data suggest that the chondrocyte can down regulate any effects on the cell, whereas, the matrix vesicle is unable to do so. In this manner, the cell is able to synthesize the matrix vesicle under genomic control and then regulate its activity later by nongenomic mechanisms.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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