Chapter 21

NEW BONE FORMATION INDUCED IN POSTFETAL LIFE BY BONE MORPHOGENIC PROTEIN

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Introduction

EVEN THOUGH a great deal is known about the morphology and the chemistry of healing fractures, no explanation has been found for the fact that bone is one of the few tissues in the mammal to regenerate completely in adult life. Regeneration in other tissues, i.e. epithelium and muscle, is only partial; in the central nervous system, it is nil. In tissues other than bone, large defects are ordinarily repaired with scar tissue. Our working hypothesis is that bone regeneration is initiated by an inductive agent stored in the structure of the organic matrix. The hypothesis is based on observations that implants of bone matrix, bone matrix gelatin, and a recently isolated bone morphogenetic protein (BMP) fraction induce differentiation of mesenchymal type cells into bone in an extraskeletal site (80, 81). Conceptually, in the adult mammal, preexisting preosteoblasts and osteoblasts are limited in number and bone tissue turnover requires recruitment of perivascular mesenchymal-type cells by a BMP to replenish bone cells lost by aging and injury.

Bone regeneration recapitulates the sequence of events of bone development in the embryo. Embryonic development occurs in two main phases, a covert morphogenetic phase and an overt phase of cytodifferentiation (87). As in embryonic bone development, the morphogenetic phase of adult bone regeneration begins with cell disaggregation, migration, and reaggregation. The cytodifferentiation phase follows by differential gene activation, synthesis of specialized cell products, and the emergence of the cartilage and bone cell phenotypes. Following bone injury or transplantation, regeneration would be initiated by release of BMP from bone matrix, which in turn institutes the morphogenetic phase of new tissue development. This is a brief summary of methods of preparation and bioassay of bone matrix, bone matrix gelatin, matrix and osteosarcoma protein fractions with BMP activity.

Experimental Observations

The differentiation of bone *de novo in post fetal life* from perivascular connective tissue cells is observed in intramuscular implants of viable and

nonviable tissues, crude fractions, and purified proteins with BMP activity. BMP may also be bioassayed in systems *in vitro*. The sources of BMP are bone or dentin matrix (5, 27, 47, 65-85), selected bone tumors (68), and isolated bone tumor cells (71). The yields of new bone from each source is measured in terms of mg of bone ash in histologically valid deposits of new bone, by levels of alkaline phosphatase activity, or by uptake of ^{45}Ca per mg of dry weight of tissue, and by other parameters. A list of yields in typical experiments on BMP activity in various systems is shown in Table 21-I.

Bone Matrix: In mice, rats, rabbits, guinea-pigs, and hamsters, *allogeneic* implants of cold HCl-*demineralized bone matrix* in tendon, muscle, subcutaneous spaces, brain, and other tissues are angiogenetic (77). The bone matrix stimulates proliferation and growth of new capillaries in and around the implant. The matrix is also bone morphogenetic and induces perivascular connective tissue to differentiate into an ossicle of cartilage and woven bone (66). In the process of remodelling of the woven bone into lamellar bone, the ossicle is colonized by bone marrow. The marrow is formed from blood-borne bone-marrow-derived stem cells in response to an osseous environment. The yield of new bone is proportional to the mass of the implanted matrix (77). *In vivo,* ossicle development proceeds to completion (Figs. 21-1 to 21-7).

Either the nutritional conditions or the vascular elements for ossicle

TABLE 21-I
YIELDS OF HETEROTOPIC BONE IN SYSTEMS IN RESPONSE TO BONE MORPHOGENETIC PROTEIN (BMP) FRACTION *IN VIVO* AND *IN VITRO*

Preparation	*Site*	*Estimates of Bone Yield mg Bone Ash/g Preimplanted Substance*
EDTA demineralized bone	muscle pouch	104 ± 40
HNO_3 demineralized bone	muscle pouch	0
HCl demineralized bone matrix	muscle pouch	454 ± 32
	subcutaneous space	487 ± 26
	anterior chamber of the eye	464 ± 32
Bone matrix gelatin	muscle pouch	504 ± 21
Dentin matrix gelatin	muscle pouch	545 ± 41
Bone matrix gelatin and muscle	diffusion chambers	195 ± 21
Bone matrix gelatin and muscle	in tissue culture	0 (cartilage only)
Collagenase released BMP	in diffusion chambers	954 ± 56
Ethylene glycol soluble BMP	in diffusion chambers	864 ± 36
GuCHl soluble BMP	in diffusion chambers	796 ± 80
Urea soluble BMP	in diffusion chambers	961 ± 59
Urea soluble BMP	insolubilized in calcium phosphate ceramic	>1000
Osteosarcoma tissue GuHCl soluble BMP	insolubilized in calcium phosphate	
Osteosarcoma tissue GuHCl soluble BMP	muscle pouch	884 ± 52

CELL POPULATIONS IN THE INTERIOR OF IMPLANTS OF BONE MATRIX IN MUSCLE

Days postop.	Area of Matrix Resorbed	%	Total cell count per high power field, in 10 typical excavation chambers	Estimate of total no. of mitotic divisions of the average ^{3}H-thymidine labeled mesenchymal cell	Estimates of differential cell count, per 10 typical high power fields
5		2	5 ± 3	0	60 Polymorphonuclear leucocytes 15 < Small lymphocytes, Plasma cells 5 Red blood cell debris 20 Ameboid mesenchymal cells 100
10		11	34 ± 8	1-2	25 Endothelial cells and RBC 25 < Plasma cells and Ameboid mesenchymal cells 50 < Multinucleated giant cells, Proliferating mesenchymal cells
15		20	108 ± 12	2-3	45 Endothelial cells and RBC 50 < Multinucleated giant cells, Proliferating mesenchymal cells, Hypertrophied mesenchymal cells 5 < Chondroblasts and cytes, Osteoblasts and cytes
20		36	282 ± 36	3-4	60 Endothelial cells and RBC 30 < Multinucleated giant cells, Proliferating mesenchymal cells 10 < Chondroblasts and cytes, Osteoblasts and cytes, Osteoclasts
40		90	1040 ± 98	4-6	75 < Bone marrow, Endothelial cells, and RBC 10 < Multinucleated cells, Proliferating mesenchymal cells, Fibroblasts 15 < Chondroblasts and cytes, Osteoblasts and cytes

Figure 21-1. Diagrams of the bone morphogenetic response to an implant of 0.6*N* HCl demineralized bone matrix in rats showing time percentage of matrix resorbed, number of newly generated host bed cells, number of mitotic divisions determined by ^{3}H-thymidine uptake, and the selection of differentiated cells at five day intervals.

formation are not provided by presently known techniques of tissue culture; in systems *in vitro,* the end product of differentiation in response of mesenchymal cells to bone matrix is always cartilage but if the explant is transplanted back into an isologous host, endochondral ossification occurs and the end product is an entire new bone (Fig. 21-7) (35). Experiments on BMP activity of matrix in tissue culture have been valuable for determination of the time intervals (35, 64) and other factors for transfer of BMP (80, 81), as well as for identification of cell populations that are competent to respond to it (40-43).

For a number of reasons, matrix-induced bone formation was suspected in 1889 (55) but not demonstrated until as recently as 1965 (65). For more than one-half century, it was common practice to demineralize bone in nitric acid. In the 1950s, EDTA became a favorite demineralizing agent. In 1965, nitric acid was found to deaminate BMP. Later EDTA was observed

to provide almost optimum conditions for digestion of a large part of the BMP by endogenous enzymes. For about three years after the discovery of matrix-induced bone formation, our working hypothesis was that either solid state piezoelectric or net surface charge effects might be sufficient to initiate differentiation of mesenchymal-type cells into bone cells (69). Variations on this theme were brought forward by others who supposed that bone collagen might be the inductive agent (25-27). Although by 1970 there was sufficient evidence to exclude the intact bone collagen molecule as the BMP, in 1974 we investigated the possibility that certain cross-links of bone collagen might emit the inductive signal. Figure 21-8 illustrates an experiment designed to investigate the bone collagen lysino-aldehyde cross-link. Table 21-II summarizes the great preponderance of evidence for and the paucity of present arguments against the concept of a noncollagenous BMP. Nevertheless, at the time of this writing, there are many references in the literature on the permissive effects of collagen on dif-

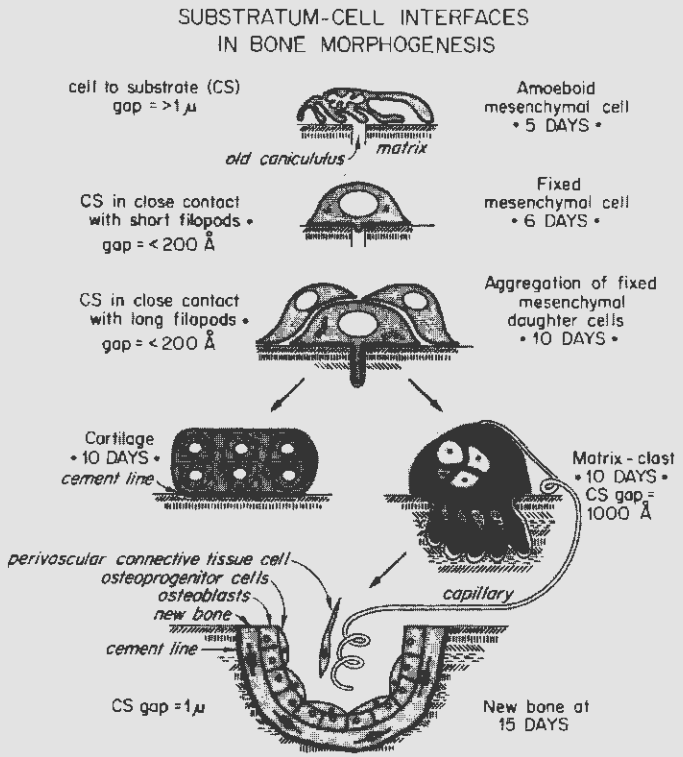


Figure 21-2. Diagrammatic representation of the interfaces between the host and donor matrix at five, six, ten, and fifteen days. A cement line forms between the new bone and the implanted matrix. Except where it comes in contact with new osteoblasts, the donor matrix does not recalcify.

TABLE 21-II
EVIDENCE FOR AND AGAINST A SPECIFIC BONE MATRIX MORPHOGENETIC PROTEIN OR PART OF A PROTEIN (BMP)

PRO

Irreversible extinction of BMP activity by:

Deamination by HNO_2 or HNO_3
Dinitrophenylation
.1*N* NaOH, 2°C, 24 hours
Ethylation by 0.x*N* HCl in 70% alcohol
25 mmoles/l $CuCl_2$, 2°C, 24 hours but not Ca, Sr, Co, or $PbCl_2$
Benzalkonium chloride (Zephiran®) [but not thiomerisol (Merthiolate®) or povidone-iodine (Betadine®) antimicrobial agents]
Bacterial infection
Delayed hypersensitivity (Cell mediated) immune response ^{60}Co irradiation 2 Mrads but not 1.0 Mrads
Limited trypic digestion at 15°C, 8 hours
Pronase
Tissue specific endogenous protease (BMPase), 37°C, 72 hours
Lathyrism
Mg deficiency
Zn deficiency

Reversible extinction by:

β-mercaptoethanol (or Diiodothreitol) reduction and oxidation
p-chloromercuribenzoic acid and cysteine
Bromodeoxyuridine after but not before 72 hours of cells on a substratum of bone matrix in culture
Explanation of osteosarcoma *in vitro* and transplantation of tissue cultures back into syngeneic species

Resistance to degradation by:

DNAases, RNAases, Neuraminidase, β-galactosidase, chondroitinases, phospholipases, lipases, collagenase

Isolated by:

Collagenase digestion; 50% ethylene glycol in phosphate buffered saline; .5*M* $CaCl_2$ in 6*M* Urea; 4*M* guanidine hydrochloride

Sources:

Dentin and bone matrix gelatins, osteosarcoma cells and tumors

CON

Bone formation occurs without bone matrix in:

Healing Achilles tenotomy in rats but not in rabbits or other species
8 percent of subcutaneous implants of glass chambers in rats
Injections of 30 ml of 70% alcohol into quadraceps muscles in 9 percent of rabbits but not rats, mice, or guinea pigs
Subcutaneous implants of calcifying polyhydroxymethylmethaerylate sponge (Hydrom) within ten weeks in young pigs but not rats or other species
Resorbed areas of old tubercles and other foci of pathological calcification in human beings
HeLa cells, amnion Fl cells, and vaccinia transformed fibroblasts

ferentiation of embryonic cells, and the implication is that bone collagen could serve as BMP (48-53). It seems more reasonable to supose that, even in embryonic development, collagen is too ubiquitous to be more than the carrier of morphogenetic molecules, and that in matrix-induced bone formation, collagen would serve as the carrier of BMP. Investigations in progress are designed to determine whether a scission product of the bone

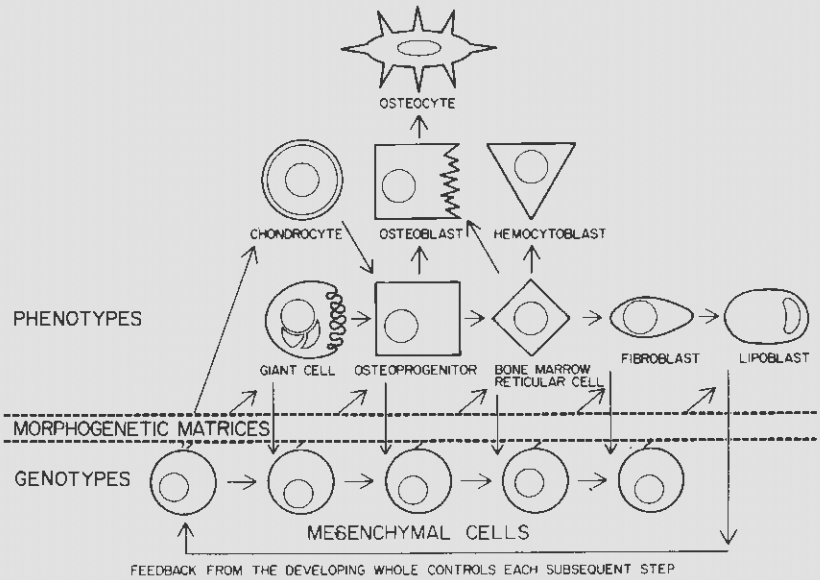


Figure 21-3. Diagrammatic representation of the derivation of specialized cells in the morphogenetic response to an intermuscular implant of demineralized bone matrix. The hypothesis is that the response is induced by allogeneic matrix but synthesized by autologous osteoblasts and successively fed back to developing cell populations to culminate in the formation of a new ossicle.

precollagen might be retained in bone matrix to function as a BMP.

Bone Matrix Gelatin: When, at 2°C, bone is demineralized in HCl, processed to sequentially extract lipid, lipoproteins, proteoglycans, sialoproteins, phosphoproteins, γ-carboxyglutamate-rich proteins, and the collagen is then converted to gelatin, the product is bone matrix gelatin (76). Implants of bone matrix gelatin are more rapidly resorbed than whole bone matrix and have a high specific activity of BMP. At 37°C, bone matrix gelatin is soluble and releases a rapidly diffusible BMP protein fraction. This fraction is soluble and diffusible in interstitial fluid because, when implanted intramuscularly inside of multiple walled diffusion chambers, bone develops on the outside (Fig. 21-9). The distance of diffusion is greater than 750 μm. The pore size for transmission of BMP is as small as 0.025 μm. These observations on bone matrix gelatin made possible the following four important advances in knowledge of BMP. BMP is not a function of the assortment of noncollagenous proteins that are soluble at 2°C in EDTA or various neutral buffered solutions. BMP is soluble in 6*M* urea at 2°C, and in tissue fluids at 37°C. The diffusion chamber method is used for bioassay and provides evidence for the concept of BMP as a diffusible molecule in interstitial fluids.

Tissue Culture: In systems *in vitro* BMP activity is assayed by explanta-

Figure 21-4. Photomicrograph of implant of demineralized bone matrix in an adult allogeneic rat, four weeks after the operation. Note: appositional metachromatic staining deposits of new bone on surfaces of pale-staining old matrix; pools of bone marrow are enclosed in the deposits of new bone.

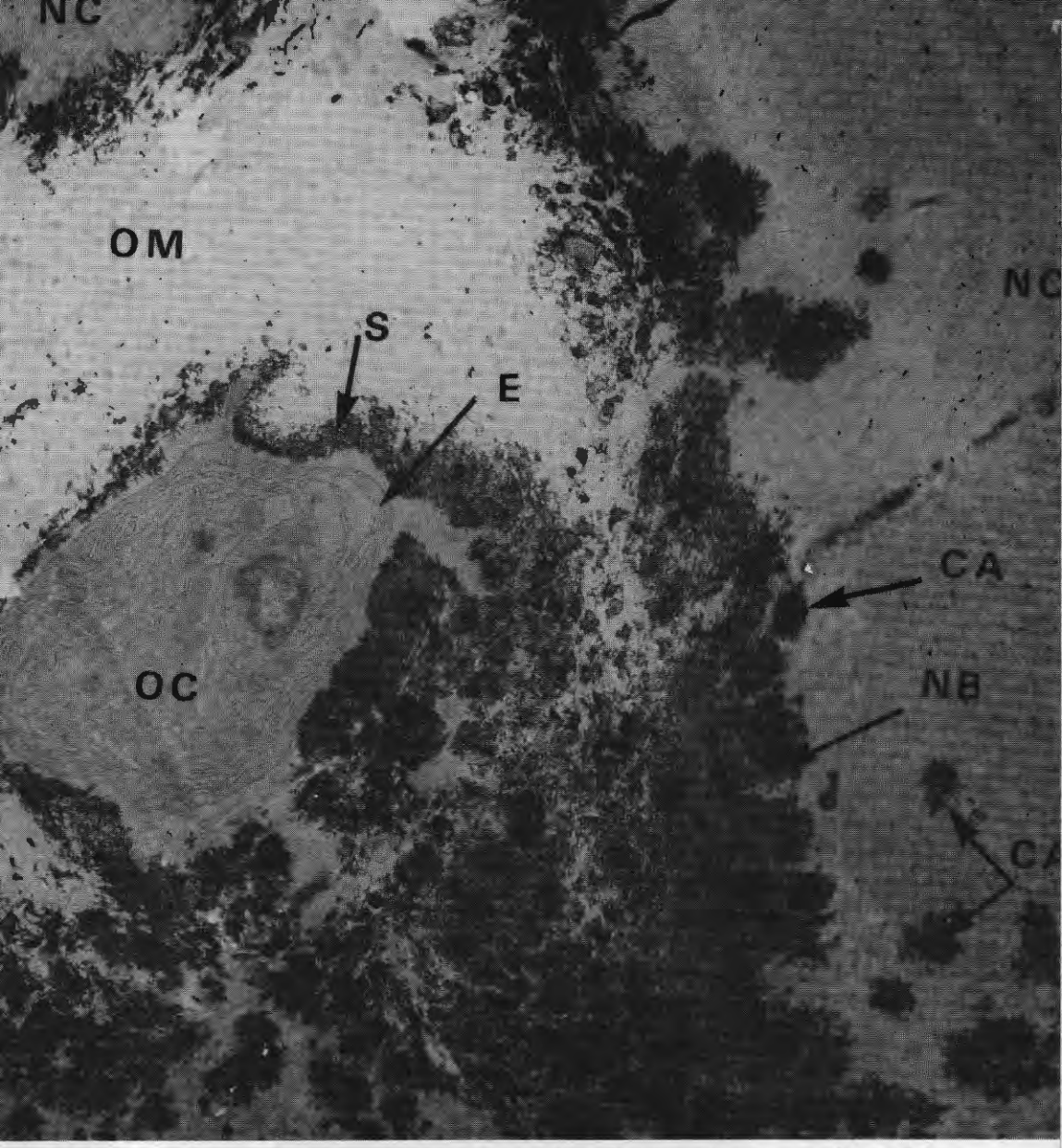


Figure 21-5. Electron micrograph of the earliest deposits of new bone on surfaces of the implanted matrix. Note: new matrix (NC); old implanted matrix (OM); deposits of mineral at the interface between osteoblasts and implanted matrix (S); osteocyte (OC); endoplasmic reticulum; new bone (NB); clusters of mineral surrounding ruptured matrix vesicles. The old matrix recalcifies only where it comes in contact with osteoblasts.

tion of cell cultures on a substratum of bone matrix or bone matrix gelatin. In response to BMP in the substratum, as noted previously, the connective tissue outgrowth of muscle differentiates into cartilage (Fig. 21-10). Bone marrow cell cultures that do not differentiate *in vitro* will differentiate into cartilage if bone matrix gelatin is present in the system. For controls, the matrix is prepared by autolytic degradation of the BMP in a buffered salt

Figure 21-6. Deposits of woven and initial colonies of bone marrow stem cells appearing during remodelling stages of formation of an ossicle.

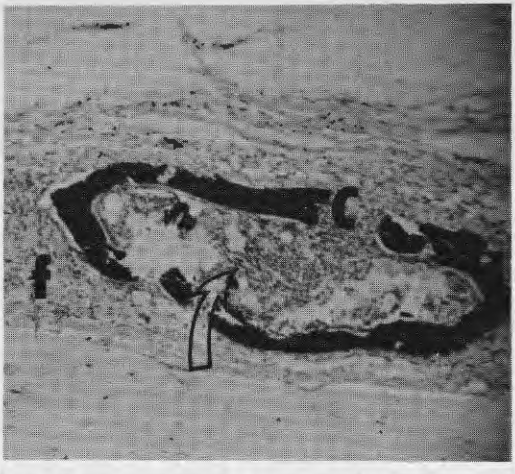


Figure 21-7. Undecalcified section of an ossicle formed from remodelling of woven bone into lamellar bone and bone marrow. Note: fibrous envelop (f); cortex of lamellar bone (c); bone marrow (arrow).

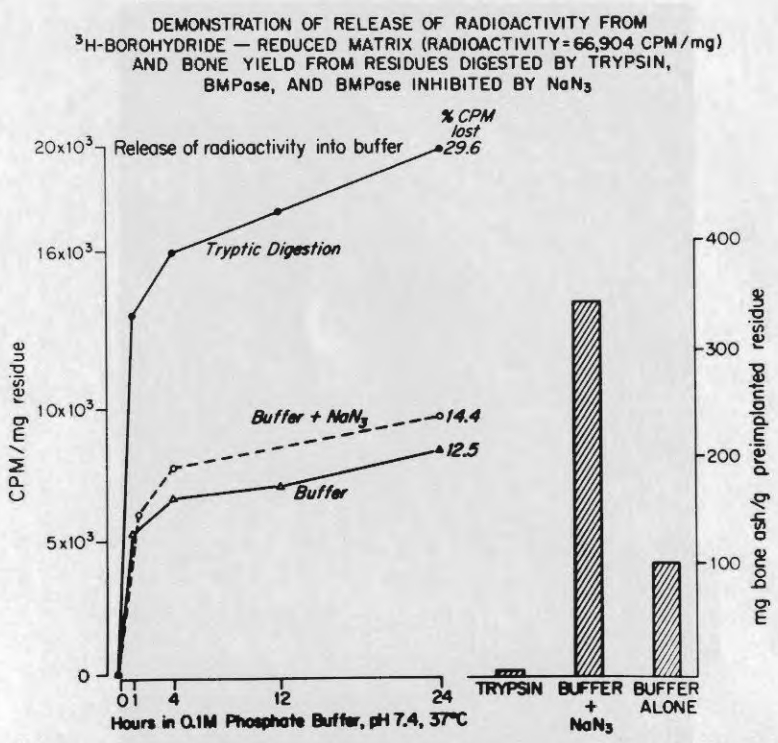


Figure 21-8. Graphic representation of relationship between bone yield and loss radioactivity by tryptic digestion of ^{3}H-borohydride-labelled bone matrix. More than 70 percent of the radioactivity is *retained* in residues after all the BMP activity is degraded. More than 80 percent is retained in residues either before or after autolytic digestion of BMP. These observations are interpreted to indicate that BMP is an entity separate from collagen or any other protein with borohydride-reducible aldehyde bonds.

solution, by BMPases that do not release hydroxyproline into the medium (Fig. 21-10). The degradation can be prevented by the addition of sulfhydryl group enzyme inhibitors, i.e. iodoacetic acid, iodoacetamide, *N*-ethyl maleimide, etc., to the buffer solution (74, 75). Many other control substrata can be prepared to eliminate BMP activity without solubilization of collagen or appreciable reduction of other noncollagenous components of bone matrix (84). Measurements of the products of differentiation *in vitro* suggest that transmission of the BMP fraction to mesenchymal-type cell receptors occurs in less than twenty-four hours (35, 85).

Collagenase Released BMP Fractions: Under conditions specified in Figure 21-11, bone matrix gelatin can be almost completely digested with purified collagenase without loss of biologic activity of the BMP fraction. The undigested substances consist of a large assortment of soluble and insoluble noncollagenous protein fractions. The most insoluble are the structural glycoproteins. Bone matrix structural glycoproteins lack BMP activity. Characteristically, structural glycoproteins are soluble in .5*M* mer-

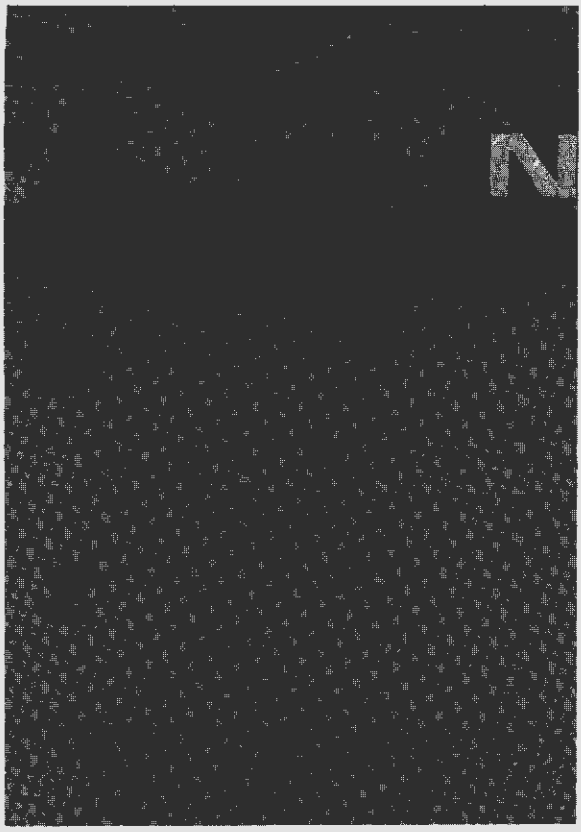


Figure 21-9. High power photomicrograph of an undecalcified section of one side of a double walled diffusion chamber. Note deposits of new bone (N), outer wall of the chamber (O), inner wall (I), and precipitate of interstitial fluid including BMP inside (bottom) and between the two walls of the chamber. Von-Kossa-hematoxylin and eosin stain.

captoethanol in 4*M* urea. BMP is soluble in 6*M* urea alone and characteristically is inactivated by mercapoethanol reduction. A collagenase digestant solution of bone matrix gelatin consists of small quantities of a large assortment of the noncollagenous proteins that are known to be found in demineralized bone matrix. The BMP activity is found in the cold water insoluble protein fraction (81). BMP is also insoluble in acids (84). The BMP fraction is trypsin labile at 15°C and almost totally destroyed in less than eight hours; it is partially resistant to pepsin, and totally resistant to chondrotinases A, B, and C, amylases, phosphatases, lipases, phospholipases, or neurimidase, but not pronase and various proteolytic enzymes (74, 75).

Non-Enzymic Methods of Separation of a BMP Fraction from Bone Matrix Gelatin: Protein fractions with BMP activity are extractable from

bone matrix gelatin at 2°C with: 50% ethylene glycol (EG) in 0.1*M* phosphate buffered saline (80); 4*M* guanidinium HCl (22, 23); .5*M* $CaCl_2$ in 6*M* urea (78). Protein fractions obtained by these solvents are bioassayed for BMP activity by implantation either in diffusion chambers in muscle pouches in allogeneic recipients or in pellets in the thigh muscles in immunodeficient athymic mice.

Fractionation of Proteins with BMP Activity: BMP protein fractions are not amenable to conventional chromatographic techniques for fractionation of globular proteins. The BMP active components either remain in the unbound fraction or adhere so avidly to .5*M* agarose and other gels that elution is possible only with 50% EG. Similar problems are encountered with ion exchange chromatography. A small quantity of BMP protein fractions bind to Con A Sepharose® and are recovered in α-MM and EG eluates (Figs. 21-12, 21-13). These protein fractions are presumed to be bound by mechanisms of carbohydrate recognition and hydrophobic interaction. Polyacrylamide SDS gel electrophoresis reveals that these fractions consist of at least several Coomassie Blue stainable components. Experiments are in progress to separate BMP from other proteins in the BMP active fraction and ascertain the homogeneity of BMP. In some respects, BMP resembles one of the many lectins that are now being found in animal (44) as well as plant tissues.

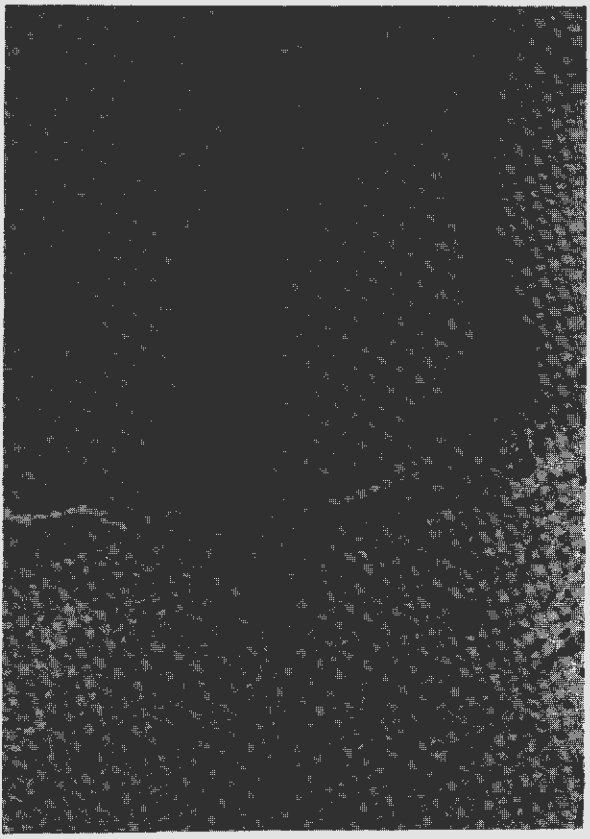
Coprecipitation with Calcium Phosphate: The number of components of a BMP protein fraction can be reduced from (five to) seven to three by coprecipitation with calcium phosphate. The BMP components are separated from the calcium phosphate within a membrane sac dialysis against .5*M* EDTA. Both coprecipitates and EDTA-released protein fractions transmit BMP activity through interstitial fluid percolating through diffusion chambers at 37°C (Figs. 21-14, 21-15). Of the three EDTA-released components, one stains densely and two only very faintly with Coomassie Blue. Investigations are in progress to ascertain whether any of the three or none, or some combination of stainable and nonstainable proteins, transmit BMP activity.

Bioassays of the calcium phosphate BMP coprecipitates, by implantation in diffusion chambers, are almost invariably positive. Outside of diffusion chambers in direct contact with the host tissues, calcium phosphates incite a chemical inflammatory reaction that blockades transfer of BMP. Experiments are in progress on BMP adsorbed to ceramic calcium phosphate.

Gelatin Peptide Chains: Bone collagen and gelatin are denatured or solubilized by all presently known enzymic and nonenzymic methods of solubilizing BMP. While the intact collagen or tropocollagen molecules are not essential for BMP activity, variable and generally very small quantities of hydroxyproline containing peptides appear in BMP protein fractions.

Figure 21-10. Photomicrograph of cartilage developed from connective tissue cell outgrowth of neonatal muscle in crevices in a substratum of bone matrix *in vitro* (right). The same outgrowth into crevices in a substratum of autolysed bone matrix differentiates only into fibroblasts (left).

Calculated in terms of collagen, these quantities could represent as little as 1 percent and as much as 40 percent contamination with collagen peptide chains, or fragments of chains. The possibility that some hydroxyproline may reside within the structure of a BMP molecule has not been excluded. Present data does not exclude the possibility of a derivation of BMP from a scission product of bone procollagen peptide, either of N or C terminus, conceivably retained in the matrix. In this respect, N terminus is interesting because it is believed to contain an OH-proline residue in its structure (46). Until BMP is purified and sequenced, it will not be possible to distinguish contaminants containing OH proline from intramolecular OH proline of BMP. The quantity of OH proline in present preparations of BMP protein fractions is only 3 to 5 μg/mg. These fractions are prepared by coprecipitation with calcium phosphate and released either in solution by EDTA or in an insoluble form by citric acid (78).



Dentin Matrix Gelatin: The above described experiments on bone matrix are entirely reproducible with rabbit dentin matrix gelatin. Theoretically, dentin should be ideal for isolation of BMP. Dentin is relatively homogenous in structure and dentin matrix proteins are uncontaminated with bone tissue cellular proteins. Membrane dialysis demonstrates that the molecular weight of BMP protein fractions derived from dentin is greater than 10,000 and the molecular mass is smaller than 50,000 (14).

BMP Protein Fractions Prepared From Osteosarcoma: Implants of freeze-dried isolated osteosarcoma cells (71) and GuHCl extracts of osteosarcoma tissue (22, 23) in pellets in the mouse thigh muscles or in diffusion chambers transmit BMP activity, the same as bone matrix and bone matrix gelatin (Fig. 21-16). By means of CsCl high density ultracentrifugation techniques, Hanamura et al. (22) obtained evidence for BMP activity in protein fractions in the range of 63,000 MW (22). Since these fractions are chemically extracted from highly cellular tumors having only an insignificant calcified intracellular matrix, and the MW may be

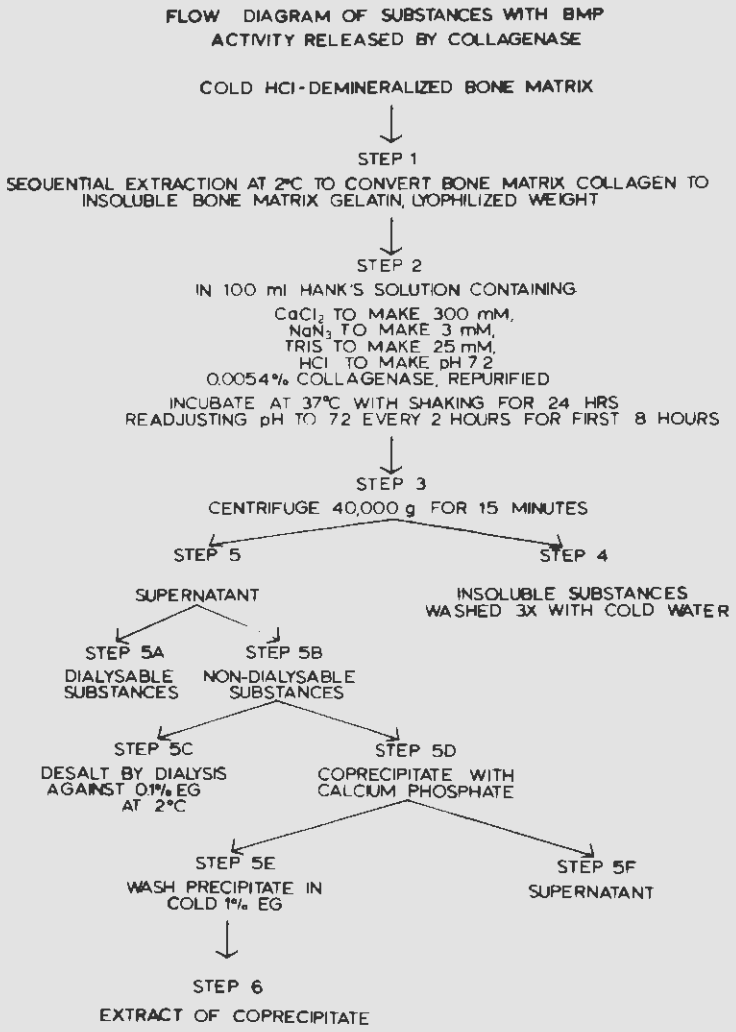


Figure 21-11. Six steps for preparation of soluble BMP from rabbit bone matrix. The high concentration of calcium ion in the digestant solution in step 2 stabilizes the structure and suppresses the degradation of BMP. Coprecipitation with calcium phosphate concentrates and purifies BMP.

as much as two times greater than that of dentin or bone matrix BMP, it is interesting to speculate about an intracellular or cell surface bound proBMP (Figs. 21-16 through 21-20). Under dissociative conditions in 4*M* guanidine HCl and with the use of sepharose 6B column chromatography, Hanamura et al. (23) obtained a BMP protein fraction with at least two distinct components, one with an assigned MW of 23,000 and another of 12,500. Further investigations are in progress to determine whether in the

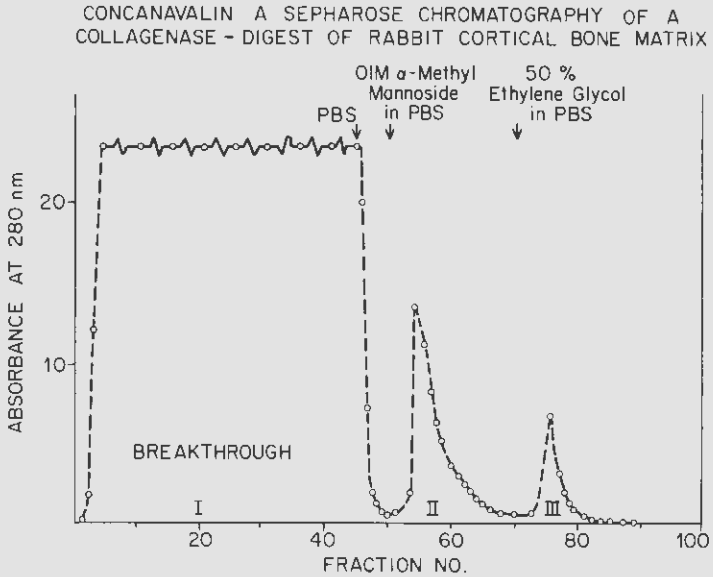


Figure 21-12. Chromatogram of a collagenase digest containing BMP. Note the same two peaks as shown in Figure 21-13. The quantity of unbound gelatin peptides in the breakthrough is enormously greater than obtained by nonenzymic chemical extractant solutions.

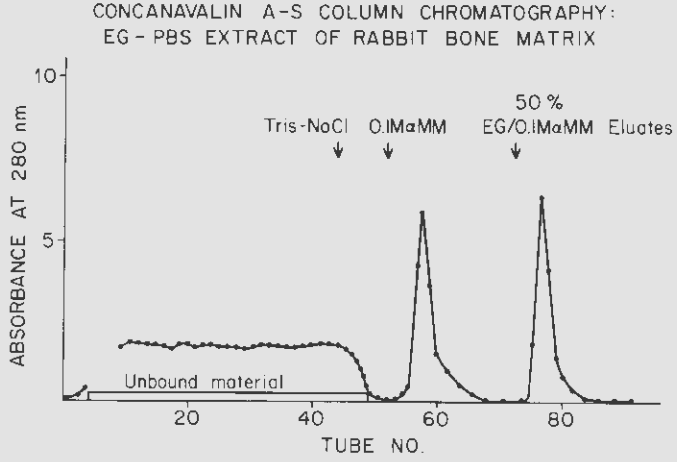


Figure 21-13. Chromatogram of a solution of proteins obtained by chemical extraction of rabbit bone matrix gelatin with 50% ethylene glycol (EG) in phosphate buffered saline. BMP is soluble in this aqueous nonaqueous solvent mixture, and partially recovered in both the α-MM and EG eluates. The gelatin peptides are separated from the BMP and found in the unbound fraction. The α-MM fraction is presumably bound by carbohydrate recognition. Theoretically, the EG eluted fraction is bound by hydrophobic interaction.

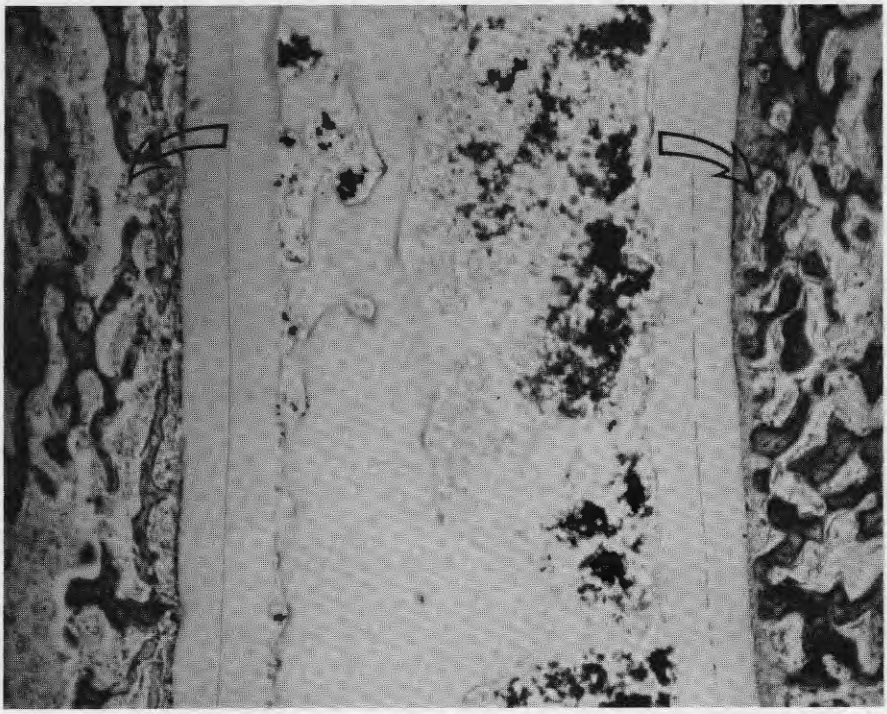


Figure 21-14. Photomicrograph of an undecalcified section of a double walled diffusion chamber containing a coprecipitate of calcium phosphate and BMP. Note large deposits of bone covering the outer surfaces (arrows). Hematoxylin, eosin, and azure stain.

native state BMP is a single homogenous molecule or a part of a macromolecule.

BMPases: The observation that BMP is spontaneously degraded within twenty-four hours after matrix is exposed to a buffer solution, pH 7.4, at 25-36°C led to the search for BMPases (74). The degradation occurs in the intact undemineralized bone from the action of endogenous neutral proteases with pH optima of 7.0 to 7.4. The mechanism of degradation is enzymic in character because it is more rapid at physiologic than at acid pH, heat inactivated in the range 40-60°C (BMP withstands temperatures of 70°C); inhibited in EDTA (pH 7.4) at 2°C but not 28°C; inactivated by sulfhydryl group enzyme inhibitors. The degradation of BMP by endogenous enzymes is as complete as with tryptic digestion at 15°C for eight hours. In both instances, the BMP is lost from the matrix without release of OH proline into the medium (75). Of particular interest, BMP activity is destroyed by incubation of BMPase-free bone matrix gelatin, by incubation *in vitro* at 37°C in a suspension of lyophilized bone powder. Incubation in suspensions of other lyophilized tissues, e.g. muscle, tendon, skin, etc.,

does not remove BMP from bone matrix gelatin (unpublished observations).

Endogenous (BMPase) enzyme degradation of BMP is not prevented by ϵ-amino caproic acid, an inhibitor of cathepsins. BMP loss is associated with the release of ^{35}S-cysteine labelled proteins of bone matrix; about 60 percent of the loss can be prevented by addition of only 3 mmoles/liter of iodoacetic acid. Release of sialic acid from bone matrix by neuraminidase had no effect on BMP activity. Extraction of soluble noncollagenous proteins from bone matrix by neutral buffer solutions alone, including Tris and EDTA at 2°C, had no effect on BMP. Exhaustive extraction of the matrix with chloroform methanol or detergents had no effect on BMP activity but did reduce BMPase activity. A special selection of twelve different sulfhydryl enzyme inhibitors, remarkably different from any other known enzyme in bone, suppresses BMPase activity. *P*-choromercuribenzoate retards BMP activity, and the retardation is reversible by the addition of a surplus of cysteine to a buffer solution. Autolytic digestion of bone matrix by BMPases occurs without any alteration of the 640 Å cross-bounded structure of bone collagen. The specificity of this enzyme system is exploited to produce a control matrix for all kinds of experiments on BMP, both *in vivo* and *in vitro* (70, 74, 75).

One of the most intriguing of all properties of demineralized bone matrix is its resistance to recalcification under physiologic conditions, neither *in vitro* nor *in vivo*. Other tissues, i.e. tendon or skin, when demineralized will remineralize under physiologic conditions. If the BMP is demineralized, then incubated in a neutral buffer to activate BMPases, and then sequentially extracted to remove soluble noncollagenous proteins, the matrix will recalcify in physiologic solutions with only 1.25 mmoles/liter of Ca^{++} and 1.0 mmoles phosphate ion (82). If the BMP is demineralized but the BMP is not first degraded by autolytic digestion and then divested of soluble noncollagenous proteins, the matrix will not recalcify. The matrix will not calcify if it is incubated in an exogenous protease such as trypsin to destroy BMP at 15°C for only eight hours before any bone collagen-derived hydroxyproline is released. These experiments suggest that BMP and various soluble noncollagenous proteins must be removed before recalcification can occur.

Recalcification of demineralized bone will not occur if an underlying calcification initiator protein (CIP), closely associated with collagen, is destroyed either by endogenous or exogenous enzymes. If CIP is preserved while EDTA soluble noncollagenous proteins are removed, the matrix will calcify. These observations suggest that BMP and CIP are attached to bone collagen in tandem. CIP is isolated from a collagenase digest of bone matrix gelatin prelabelled with ^{35}S-cysteine. Whole muscle

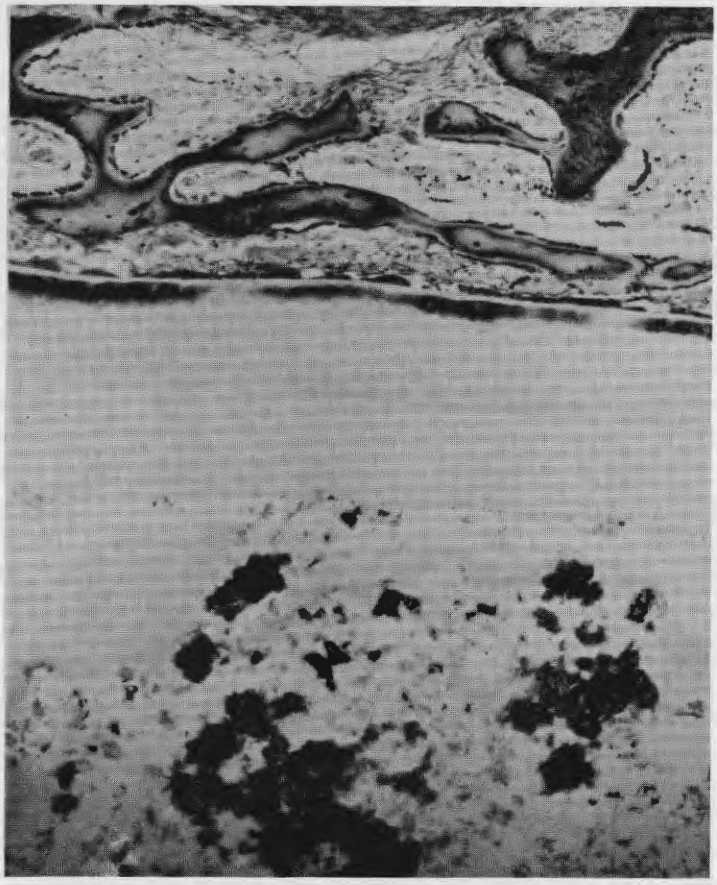


Figure 21-15A. High power view of a double walled diffusion chamber containing coprecipitate of calcium phosphate and BMP (bottom). Note: deposits of new bone covered with osteoblasts with calcified material extending inside of the surface of the outer membrane (top); hematoxylin stained calcium phosphate (bottom) surrounded by coagulated interstitial fluid. Hematoxylin, eosin and azure stain.

or rat tail tendon acid soluble collagen reconstituted in a solution of CIP promptly recalcifies. Whether CIP is the agent involved either in the process of mineralization or in pathological calcification warrants investigation.

There is no tissue in the body that is not known to calcify under pathological calcification. Tendon, however, is a favorite tissue for biochemical investigations on calcification *in vitro*. Whole tendon does not calcify, but acid-demineralized tendon and acid-soluble reconstituted rat tail tendon collagen calcifies in metastable solutions of calcium and phosphate ions. The obvious assumption is that calcification inhibitor substances are acid soluble and removed by demineralization. The literature on calcification

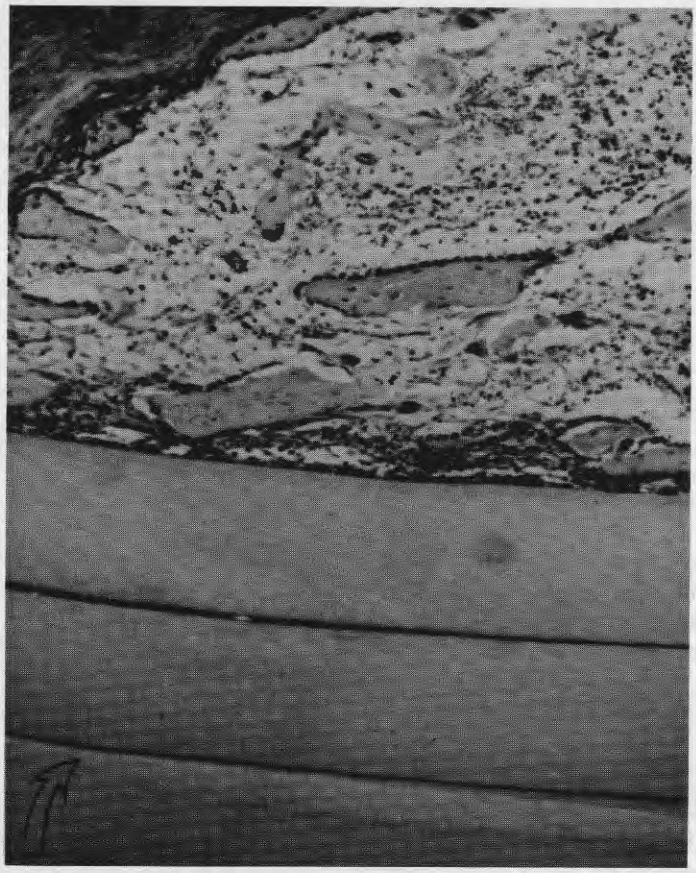


Figure 21-15B. Photomicrograph of deposits of new bone on day 21 after implantation of purified rabbit bone matrix BMP (arrow) in a double-walled diffusion chamber.

in the normal physiology of both extraskeletal as well as skeletal tissues has been reviewed in detail (67). The following substances are listed as inhibitors: bone EDTA soluble proteoglycans, phosphoproteins, sialoproteins, glycoproteins, lysoproteins, gla proteins, and other substances. However, there is some controversy about inhihitors that are considered as initiators by some observers (39, 67, 86).

HETEROTOPIC BONE FORMATION BY EPITHELIAL-MESENCHYMAL CELL INTERACTIONS: In over fifty years of research, not a single clue has been found for the nature of the biochemical control mechanism of epithelial-mesenchymal cell-induced heterotopic bone formation. Anderson (3) critically reviewed the recent literature. Uroepithelium, HeLa cells, and Fl amnion placental cells consistently induce formation of bone in muscle. Heterotopic ossification is not an unspecific response to the pressure of

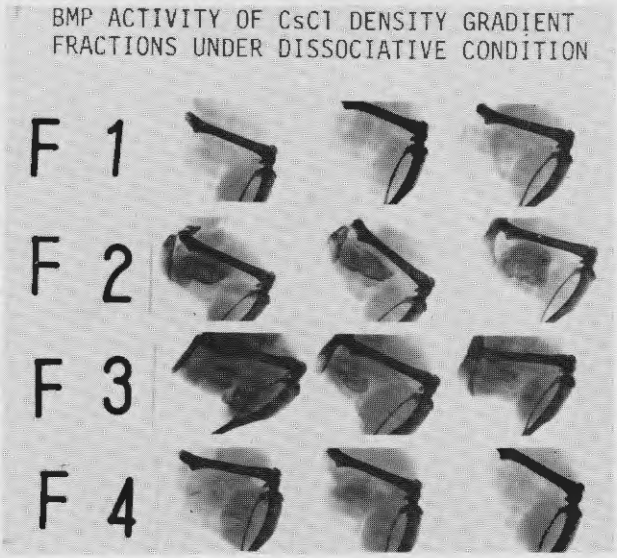


Figure 21-16. Roentgenograms of mouse thighs implanted with pellets of guanidine HCl soluble BMP prepared from Dunn mouse osteosarcoma. Note the deposits of bone produced by implants of fractions F2 and F3. (From Hanamura et al. *Clinical Orthopedics,* in press, 1981, with permission of J. B. Lippincott Co., Philadelphia.)

tumor growth because not all epithelial tumors, not even all bone tumors, evoke differentiation of host bed mesenchymal cells into bone (5, 73). The new bone differentiates in connective tissues surrounding living epithelial cells but only if the cells are transplanted into muscle, and rarely if ever in a subcutaneous site. Other tissues, i.e. bone tumor cells and normal bone matrix, induce bone formation even after freeze-drying and even in subcutaneous host tissue sites (65, 73). With the discovery of BMP in normal bone matrix and a biochemical approach to the subject of heterotopic bone formation, the important question is what are local and systemic factors common to both epithelial and BMP induced heterotopic bone formation? Patently, do epithelial cells secrete a BMP?

Summary

The observation that a specified preparation of bone matrix (65, 76) induces adult mesenchymal type connective tissue cells to differentiate in cartilage and bone has now been confirmed by over thirty different research groups (1-4, 6-23, 25-38, 40-43, 45, 47-85). The inductive agent is a BMP. The evidence for and against the BMP hypothesis is summarized in Table 21-II. While a specific BMP molecule has not yet been fully characterized, recently reported preparations consist of BMP protein fractions that are about 90 percent purified.

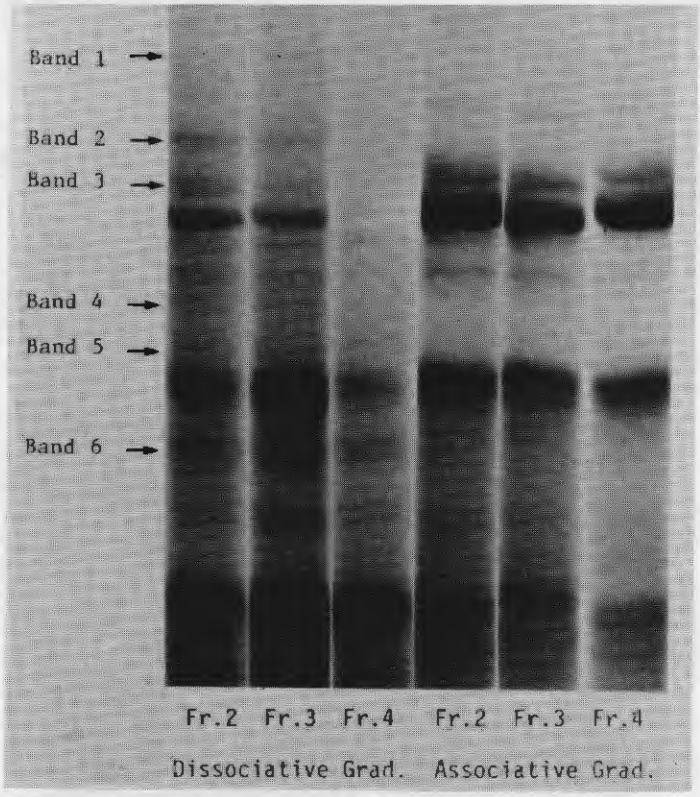


Figure 21-17. Photograph of SDS polyacrylamine gel electrophoretic patterns of the BMP active fractions. Band 4 is present only among the components of fractions 2 and 3. Under associative conditions, the component represented by band 4 combines with other components in fractions 2 and 3. (From Hanamura et al., *Clinical Orthopedics*, in press, 1981, with permission of J. B. Lippincott Co., Philadelphia.)

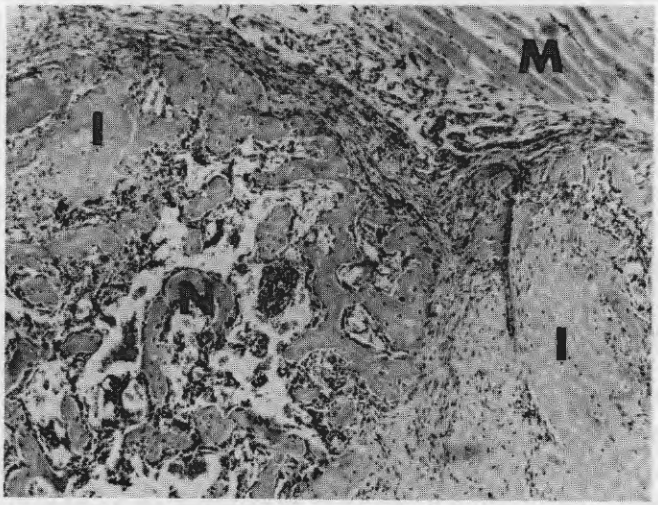


Figure 21-18. Photomicrograph of bone deposits produced by implant of fraction 2 in mouse thigh shown in Figure 21-16. Note: muscle (M), implant (I), and reactive new bone (N).

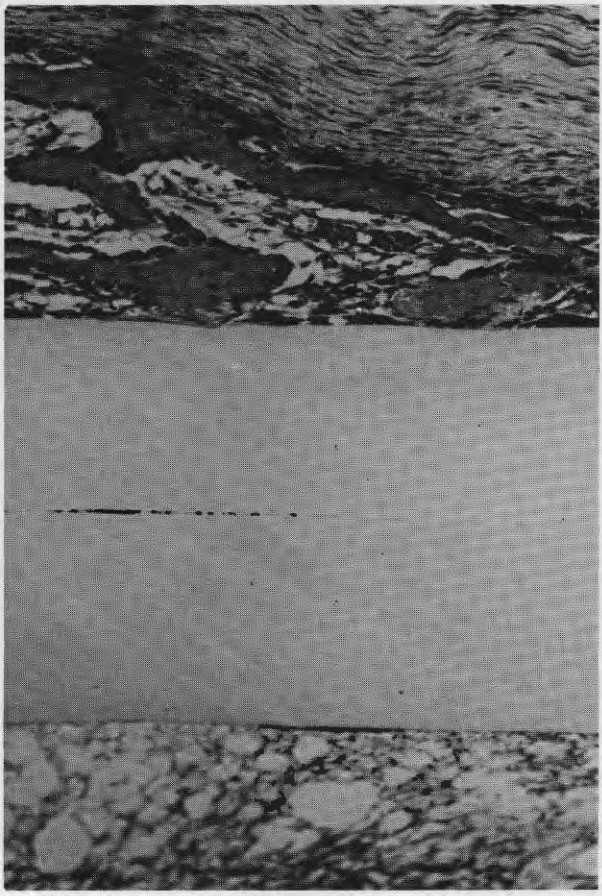


Figure 21-19. High power photomicrograph of deposits of new bone (top) on the outer surface of a double-walled diffusion chamber filled with 2 mg of osteosarcoma BMP.

BMP is insoluble in cold water at low temperatures and in acids at room temperature. BMP is soluble in 50% ethylene glycol, 6*M* urea, or 4*M* guanidinium hydrochloride. Solid support structure of the proteins, e.g. cell membranes or collagen, may serve to sustain biologically active conformation of BMP. In interstitial fluid at 37°C, BMP dissociates from solid support structures, diffuses through cellulose acetate membranes to mesenchymal cell plasma membrane receptors, and by some unknown mechanism induces differentiation of bone. BMP is a low molecular mass molecule. It can be transferred through dialysis membranes of pore size as small as 0.025 μm (250 Å). BMP is like α_2HS (4) insofar as it accumulates in cortical bone. Coprecipitates of BMP and calcium phosphate induce new bone formation with a very high incidence of positive results.

BMP protein fractions of molecular weights of 10 to 50,000 are obtained

from organic matrix of cortical bone and bone tumors (22, 23, 63, 68, 71, 73, 79). That BMP is found also in normal osteoblasts is likely but not yet proven. Whatever may be the tissue of origin of BMP, at the present time information is lacking about whether BMP is a single molecular species, a complex of molecules, an appendage of collagen, or a scission product of precollagen.

Working independently, two research groups simultaneously reported on a guanidine HCl method extraction of a BMP from osteosarcoma (22, 23, 63). Hanamura et al. (22), in the basis of circumstantial evidence from CsCl high density ultracentrifugation, isolated a protein fraction with BMP activity and a MW of 63,000. Later Hanamura et al. (23) produced subfractions of osteosarcoma BMP and proposed that monomeric forms

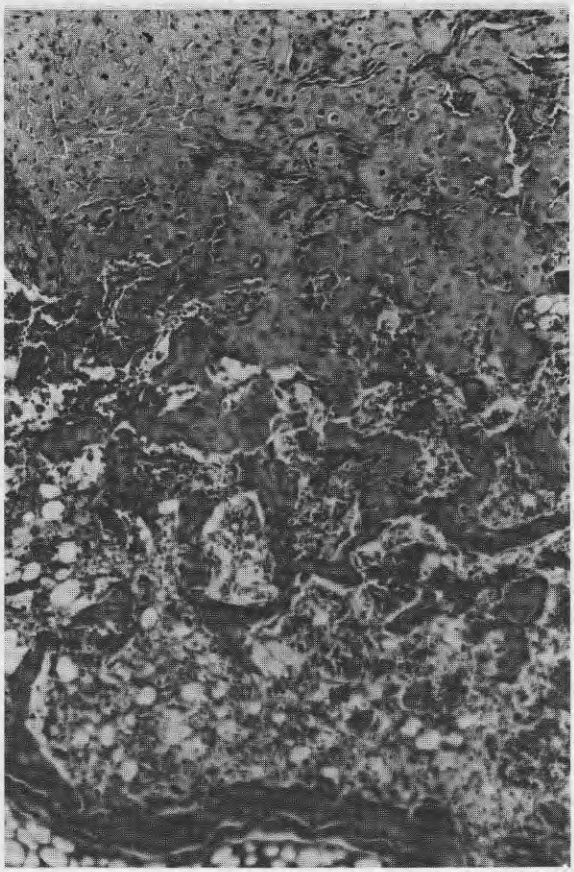


Figure 21-20. Deposits of new bone produced by implant of guanidine HCl extract of bone matrix gelatin prepared from *human* bone and bioassayed in the high muscles of an athymic mouse. Note chondroosteoid (top) and new bone including bone marrow (bottom). Undecalcified section; hematoxylin, eosin, and azure stain.

with MW of less than 30,000 transmit a high level of osteosarcoma cells that transfer a trypsin-labile BMP to host mesenchymal-type cells, which respond by differentiation into normal bone. Bauer and Urist (5) recently prepared a BMP from human osteosarcoma, by means of the guanidine HCl extraction method, and bioassayed it in athymic mice. These and detailed observations on experimental bone tumors suggest that the formation of normal bone containing bone marrow on the periphery of an osteosarcoma is an inductive response to BMP secreted by osteosarcoma cells (68, 79).

A positive bioassay for BMP protein fractions consists of formation of cartilage or bone or both in a heterotopic site. BMP induced heterotopic bone formation is influenced by the host bed responding cells, the species, and general nutritional factors including hormones, metabolites, aging. One of the most responsive connective tissue cells in long-lived animals, e.g. dogs, monkeys, and human beings, is found in bone marrow stroma. In various pathological conditions in human beings, muscle-derived connective tissues differentiate into bone in response to interaction of undefined systemic and local factors. Whether these factors include a BMP remains to be proven.

A physiologic role of BMP is suggested by experiments on lathyritic, hypophysectomized, and aged animals. BMP is deficient or absent from the matrix of lathyritic bones. It is also deficient in the matrix of hypophysectomized rats but can be partially replaced by treatment with bovine growth hormone (62). BMP activity in the matrix of senile rats and rabbits is very low. Whether BMP might contribute to the rapid rate of fracture healing in young compared with aged individuals is an interesting possibility. The question about whether a systemic circulating BMP is involved in the normal physiology of bone has not been answered by present research on heterotopic bone. When it is more fully characterized and available for parenteral administration, research on systemic effects of BMP will be possible. BMP research is bound to improve present understanding of localized mechanisms of bone formation in health and disease, and eventually become applied to the problem of repair of large bone defects from injury, infection, neoplasm, and congenital abnormalities.

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