Recombinant Human Bone Morphogenetic Protein (rhBMP) Induced Heterotopic Bone Development *In Vivo* and *In Vitro* (43970)

HOLLY VOLEK-SMITH AND MARSHALL R. URIST[1]
UCLA Bone Research Laboratory, Los Angeles, California 90024

Abstract. ***In vivo,*** **recombinant human bone morphogenetic protein (rhBMP-2) with deactivated bone matrix as a carrier, implanted in muscle in adult rats, induced development of heterotopic bone, including bone marrow. The volume of bone was proportional to the dose of rhBMP-2 in a range of 0.2–150 μg.** ***In vitro,*** **in response to 50-μg dose range, subcutis- and brain-derived outgrowths differentiated into loosely woven connective tissues composed of spindle-shaped fibroblasts, adipocytes, and cartilage. Muscle-derived connective tissues cultivated first in culture media supplemented with 50 μg of rhBMP-2 for 72 hr, then enclosed in a diffusion chamber, and immediately transplanted into a rectus abdominous muscle pouch in an autogenic rat for 28 days, induced cartilage development on the inside and transmembrane hetertoptic bone development including bone marrow on the outside. These experiments are interpreted to show that muscle derived connective tissue cells have the competence of embryonic cells to develop *de novo* in response to BMP in postfetal life.**
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Heterotopic bone development has been reported in almost every tissue in the body, but most frequently in muscle. Previous communications demonstrated differentiation of muscle-derived mesenchymal type cells into cartilage in response to bone matrix (1–15), a partially purified bone morphogenetic protein (16–21), or recombinant BMP (22, 23). Recent reports presented the response of several established cell lines (23–27) to recombinant human bone morphogenetic protein (rhBMP-2). The cells were not derived from muscle but from bone marrow or bone tissues, and possibly were determined or protodifferentiated with respect to skeletal tissue differentiation. Consequently these established cell lines were not as applicable to the problem of heterotopic bone development as primary cultures of muscle-derived connective tissues. Primary cultures of muscle-derived connective tissues, which in the lifetime of the animal normally would not differentiate into cells other than fibroblasts, were more directly applicable to the problem of heterotopic bone formation (21). We report here on muscle-derived cells before and after exposure to rhBMP-2, with and without transplantation into diffusion chambers in the anterior abdominal wall. To determine whether cells derived from other tissues were more or less competent to respond to BMP, the experiments were repeated on connective tissue outgrowths of subcutis and brain. To consider the possibility that experimental results may vary with variations in conditions of cultivation, we compared the composition of CMRL-1066, the medium employ in this study, with the composition of three other standard culture media employed in our previous investigations (2–4).

Materials and Methods

Deactivated Rat Bone Matrix. Deactivated matrix was prepared from diaphyseal segments of adult male Sprague-Dawley rat bones as follows. Long bones were cleaned, demarrowed, washed overnight in water at 4°C, and delipidized in 1:1 chloroform and methanol at 4°C for 1 hr. To digest cell remnants, the delipidized bone was autolyzed by the action of en-

[1] To whom requests for reprints should be addressed at UCLA Bone Research Laboratory, Rehabilitation Center, Room A3-34, 1000 Veteran Avenue, Los Angeles, CA 90024.

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dogenous proteases in 0.1 *M* sodium phosphatate buffer at 37°C (pH 7.2) for 72 hr and washed in distilled water. The cell free bone was demineralized in 0.6 *N* HCl at 4°C for 24 hr and then washed. To ensure complete removal of all inductive activity attributable to endogenous bone morphogenetic protein the cell-free matrix was also extracted with 0.4 *M* GuHCl in 0.5 M $CaCl_2$ at 4°C for 48 hr. The residual matrix was washed with distilled water, frozen, and lyophilized (14). Implants in allogeneic rats showed complete obliteration of all osteoinductive activity in the residual matrix.

BMP Bioassay in Systems *in Vivo*. Twenty 2-ml Eppendorf tubes were loaded with 25 mg of pulverized deactivated rat matrix. Ten tubes of matrix were wetted with 200 μl of 1–150 μg of recombinant human BMP-2 (rhBMP-2, Batch #IDIIJ021, with albumin as carrier, Genetics Institute, Cambridge, MA). Sterile distilled water (Bio-Whittaker, Inc., Walkersville, MD) was added to each tube to a volume of 200 μl. To precipitate the proteins onto the bone matrix, 0.5% (1 μl) trifluoroacetic acid was added. For controls, 10 other tubes of the deactivated matrix were immersed in 200 μl of sterile, distilled water, and 0.5% trifluoroacetic acid without rhBMP-2. The matrix absorbed the 200 μl of solutions within 2 hr. When completely dry, the matrix composites were placed in No. 3 gelatin capsules (pre-sterilized in chloroform vapors overnight) and implanted into the abdominal muscles of 10 adult Sprague-Dawley rats. To ensure internal consistency, the experimental and control implants were implanted on the contralateral sides in the same host. The anterior abdominal walls were excised, examined by x-ray, and immediately fixed in neutral formalin for histological sections.

Tissue Culture Substrata. Deactivated rat bone matrix was cut into hemicylinders 8 mm in length. The hemicylinders were sterilized in chloroform vapors overnight. The media used for all cultures were prepared by adding 15% fetal calf serum to a culture medium named CMRL (28) (Gibco, Grand Island, NY), with 1% penicillin-streptomycin (5000 units penicillin, 5000 μg streptomycin, Gibco), and 3% $NaHCO_3$ to bring the pH to 7.0–7.2. The culture system consisted of a culture dish, wire grid, and substrata consisting of either deactivated rat bone matrix hemicylinders or 13-mm cellulose acetate membranes coated with a concentrated solution of fibronectin (Sigma Chemical Co., St. Louis, MO) as described in previous publications (9, 17, 28).

Connective Tissue Outgrowths of Muscle, Brain, and Skin on Cellulose Acetate Membranes; Special Precautions for Inclusions of Tendon Attachments and Periosteum. In dissections of near-term female Sprague-Dawley rats, 0.5 mm^3 of the triceps brachialis muscle belly was excised and minced in a 20-μl drop of CMRL-1066 culture medium on a glass petri dish (see Table I below). Forty-eight explants of minced muscle was cultivated on cellulose acetate membranes (Millipore, Marlborough, MA), pore size 0.45 μm, coated with fibronectin (Sigma). For 21 days, six explants of brain tissue removed from the cerebral hemispheres with a small curette, and six explants of subcutis fragments were similarly cultivated on cellulose acetate membranes. The media were supplemented with 1–50 μg of rhBMP-2 in the culture media. Three explants of each tissue were cultivated in unmodified media. Special precautions were taken to avoid the origins and insertions of tendon attachments to periosteum and bone. The muscle belly only was considered a valid source of connective tissue cells which in the lifetime of the rat is undifferentiated with respect to cartilage and bone.

Dose-Response Bioassays. The reactions of connective tissue and growths to rhBMP-2 were measured by [^{3}H]thymidine incorporation into DNA and [^{35}S]sulfate incorporation into GAG were measured by means of previously reported methods (6, 10, 17). Graduated doses, ranging from 1 to 50 μg/25 mg of rat deactivated matrix, were implanted in triplicate in the rectus muscles of adult Sprague-Dawley rats. For assays *in vitro* neonatal muscle explants onto deactivated rat bone matrix hemicylinders were exposed to 10 μg, 1 μg, and 200 ng of rhBMP-2 per 2 ml CMRL-1066 media on Day 1, 2, and 4 (three cultures of each concentration). On Day 6, the supplemented media were replaced with media without rhBMP-2 and then changed every other day for 14 and 21 days. Explants of minced neonatal rat muscle cultivated in triplicate on cellulose acetate membranes (Millipore), were exposed to graduated doses of rhBMP-2 per 2 ml CMRL-1066 media on Day 1, 2, and 4 of culture (0.2, 1.0, 5.0, 10.0, 25.0, and 50.0 μg/2 ml of CMRL). The media were changed on the third culture day and every other day. After 10 days of cultivation, [^{35}S]sulfate and [^{3}H]thymidine were added to the media for 4 hr. Three cultures were also fixed in formalin for histological sections on Day 14 to 21 of cultivation.

Sequential Exposure of Muscle to rhBMP-2 *in Vitro* and Transplantation *in Vivo*. Diffusion chambers (Millipore) were assembled using two 0.45-μm–pore size membranes by methods previously described in detail. Six chambers with muscle enclosed, were placed on a wire grid, in a Falcon culture dish, containing 2 ml CMRL-1066 media in the inner well, and 3 ml of sterile water in the outer well. The media contained doses ranging from 1 to 10 to 50 μg of rhBMP-2 for 24 hr (one exposure), 48 hr (2 exposures), and 72 hr (three exposures) using two chambers for each time period. For controls, two chambers were exposed to media alone for 48 hr. After each time period, the chambers were transplanted subcutaneously into syngeneic rats for 4 weeks. After 4 weeks, the chambers

and surrounding tissues were excised and examined by radiographic and histological methods previously described in the literature (15).

Results

Bone Matrix Composites *in Vivo*. Composites of rhBMP-2 and deactivated rat bone matrix induced differentiation of calcifiable new bone. The incidence of heterotopic bone formation in the rectus abdominous muscle varied with the quantity of rhBMP in the composite. With lower dose levels, the incidence was 25%–50%. With higher dose (>10 μg) levels, the incidence was about 50%–100%. Figure 1, A and B show typical radiographic and histological findings. As the quantity of rhBMP increased from 1 to 150 μg, the area of computer image analyzed radiopaque bone increased from approximately 5 mm^2 to approximately 90 mm^2 (Fig. 2); 200-ng doses induced only microscopically visible deposits of new bone. Alkaline phosphatase activity on day 21 was elevated only in implants with heterotopic bone.

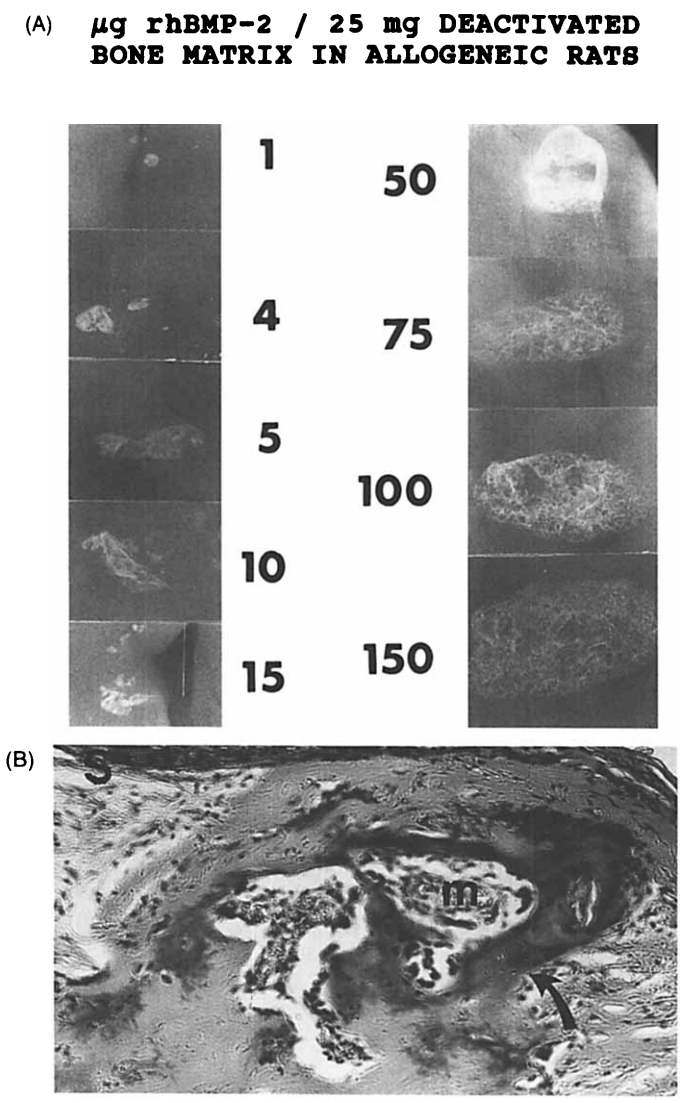

Figure 1. (A) Radiographs of ossicles developed in response to intramuscular implants of a composite of rhBMP and deactivated bone matrix carrier. Note the increase in bone mass in proportion to dosage ranging from 1 to 150 μg/25 mg of carrier. (B) Photomicrograph of bone development (arrow) in an implant of rhBMP-2 adsorbed to deactivated bone matrix after 28 days in the rectus abdominous muscles (S) in an allogenic rat. M, marrow; S, host bed muscle.

Differentiation of Cartilage *in Vitro*. When fetal rat muscle was cultivated on deactivated rat bone matrix on Day 1, 2, and 4 in culture media supplemented with 1–10 μg rhBMP-2, hyaline cartilage developed on and between bone matrix surfaces (Fig. 3, A and B). Cartilage developed in 100% of eight cultures exposed to 10 μg of rhBMP-2 and in 85% of exposed to 1 μg rhBMP-2. Cartilage did not develop in four cultures in media supplemented with only 0.2 μg of rhBMP-2.

Connective Tissue Outgrowths onto Cellulose Acetate Membranes. Table I summarizes observations on primary cultures of connective tissue outgrowth of fetal rat muscle onto a substratum of cellulose acetate membranes (Millipore), consisting of a monolayer of mesenchymal type cells and fibroblasts. In response to media supplemented with rhBMP-2, a multilayer of hypertrophied connective tissue cells, fibroblasts, and osteoid tissue developed after 14 days of cultivation (Fig. 4, A and B). Deeply stained eosinophilic osteoid with a central core of calcification appeared when the rhBMP-2 concentration was increased from 1 to 50 μg. The cross-sectional area of

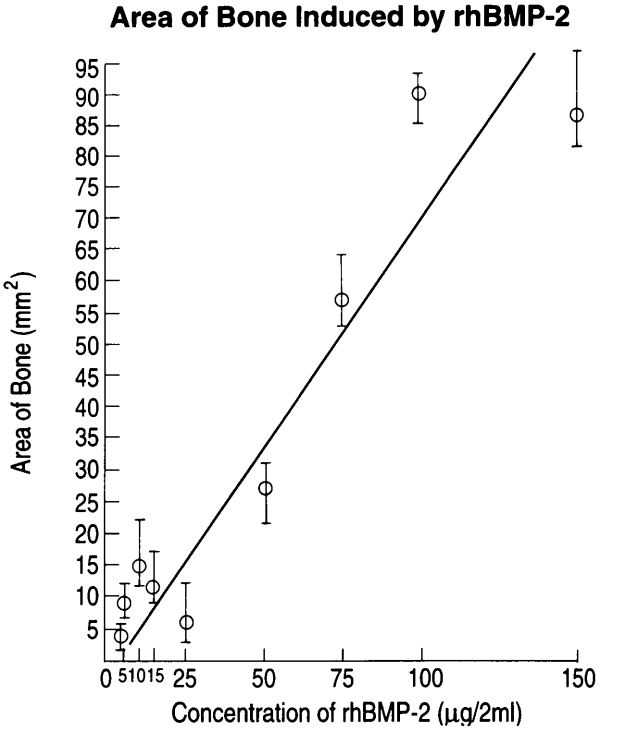

Figure 2. Graph showing the linear relationship of area of new bone and the quantity of rhBMP-2 adsorbed to deactivated matrix implanted in an allogenic rat.

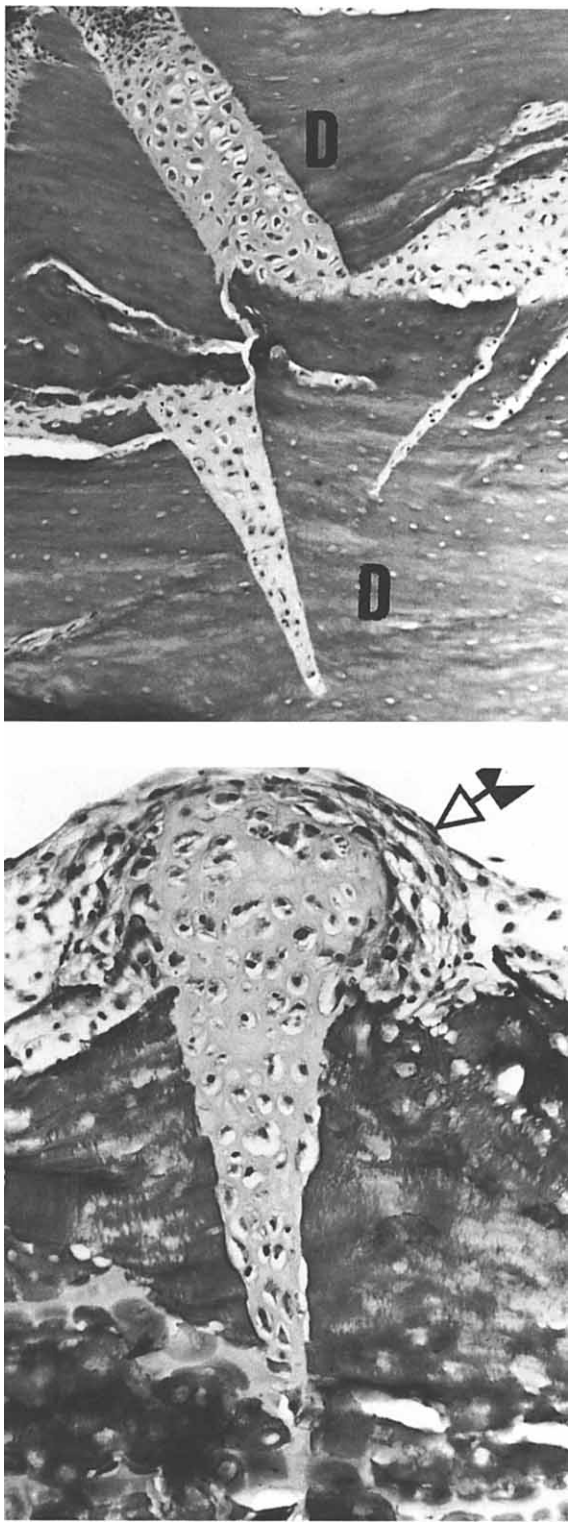

Figure 3. (A) Photomicrograph of hyaline cartilage developed within 14 days in a connective tissue outgrowth of an explant of neonatal rat muscle onto a substratum of deactivated rat matrix (D), in response to culture medium supplement with 1 μg of rhBMP-2 on days 0, 2, and 4. Stain: hematoxylin eosin and azure; magnification: ×120. (B) High-power view of cartilage derived neonatal muscle connective tissue cultivated in a furrow in a substratum of deactivated rat bone matrix on Day 14. The culture medium was supplemented with 1 μg of rhBMP-2 on Day 2 and 4. Note chondroosteoid and perichondrium (arrow). Stain: hematoxylin eosin and azure II; magnification: ×200.

Table I. Neonatal Rat Muscle Connective Tissue Outgrowths on a Substratum of Cellulose Acetate Membrane in Media Supplemented with rhBMP

Tissue of origin	Dose of rhBMP-2 (μg/2 ml media)	n^a	Incidence of cartilage, osteoid, or bone development (%)	Alkaline phosphatase (U/μg of DNA-P)[b]
Muscle	0.2	4	0	12.2 ± 0.8
	1.0	6	15	42.4 ± 0.8
	5.0	4	25	56.6 ± 1.2
	10.0	4	100	106.2 ± 5.8
	25.0	8	75	96.3 ± 4.8
	50.0	12	50	90.6 ± 5.4
Skin	1.0	6	50	15.0 ± 4.5
	5.0	6	50	21.0 ± 5.5
	50.0	6	66	30.1 ± 5.0
Brain	1.0	6	12	10.1 ± 4.0
	5.0	6	33	15.0 ± 4.1
	50.0	6	50	65.0 ± 9.5

[a] *n*, number of viable explants.
[b] Mean ± SD based on average of six explants.

osteoid in the center of the implants was logarithmically proportional to the concentration of rhBMP-2 (Fig. 5); 200 ng produced fibrous connective tissue only. As the concentration of rhBMP-2 increased from 1.0 to 50 μg, the area of osteoid grew from 3.8 to 18.2 mm^2 (Fig. 6). Alkaline phosphatase levels increased four to ten times above control. These cultures with medium CMRL-1066 produced a higher percentage of positive results than we and others (1–4) reported previously experiments using other media listed in Table II.

[^{3}H]Thymidine Incorporation into DNA. Figure 7 illustrates an increase in DNA synthesis (measured by [^{3}H]thymidine incorporation) in response to a pulse infusion of various doses of rhBMP-2 on the 10th day of cultivation. With 10 μg, there were peak levels of incorporation. With 25 μg, the level was only one third of peak levels. With 50 μg, a second measurable increase about 10% of the highest peak values of incorporation of [^{3}H]thymidine into DNA. These data suggest that rhBMP may inhibit mitosis and enhance cell differentiation.

[^{35}S]Sulfate Incorporation into GAG. As shown in Figure 8, sulfate incorporation into GAG declined when cultures were exposed to 1 and 5 μg of rhBMP-2. In response to 10 μg, [^{35}S]sulfate incorporation into GAG production increased nearly four times above baseline. With 25 μg incorporation into GAG decreased about 3-fold. With 50 μg, incorporation was about 15% above baseline, matching the shape of the curves describing [^{3}H]thymidine incorporation.

Exposure to rhBMP-2 *In Vitro* and Cultivated *In Vivo*. When the cellulose acetate chambers containing weanling rat muscle that had been exposed to 50 μg of

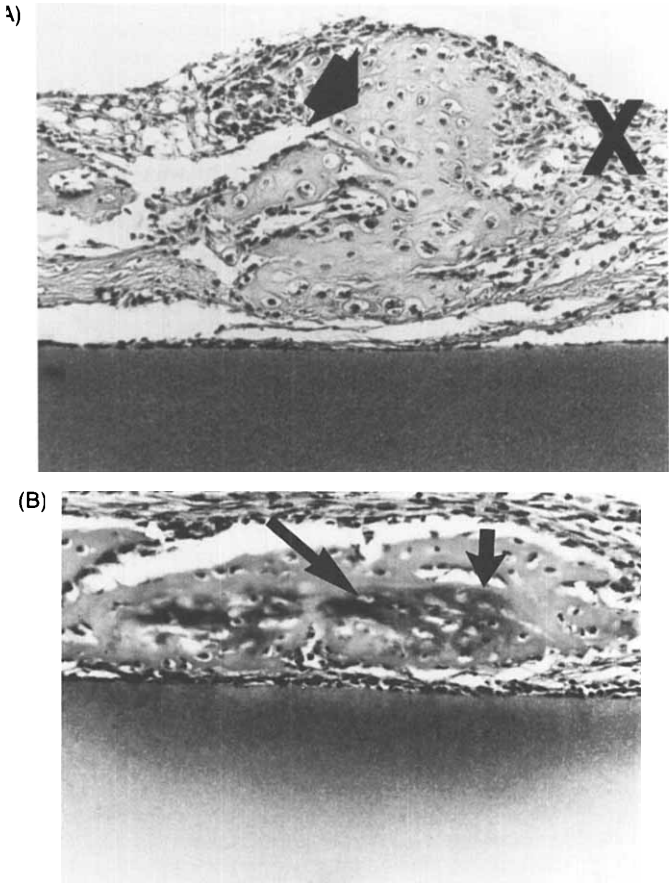

Figure 4. (A) Photomicrograph of chondroosteoid developed in connective tissue outgrowth of neonatal muscle onto a fibronectin-coated substratum of cellulose acetate (bottom), in a culture media CMRL supplemented with 50 μg of rhBMP-2 on Day 0, 1, and 2, 21 days of cultivation. X, connective tissue outgrowth of muscle; arrow, chondroosteoid. (B) Photomicrograph of calcified osteoid (arrows) developed on a substratum of cellulose acetate (bottom) on Day 14 of cultivation. Undecalcified section. Stain: VonKossa hematoxylin and eosin; magnification: ×100.

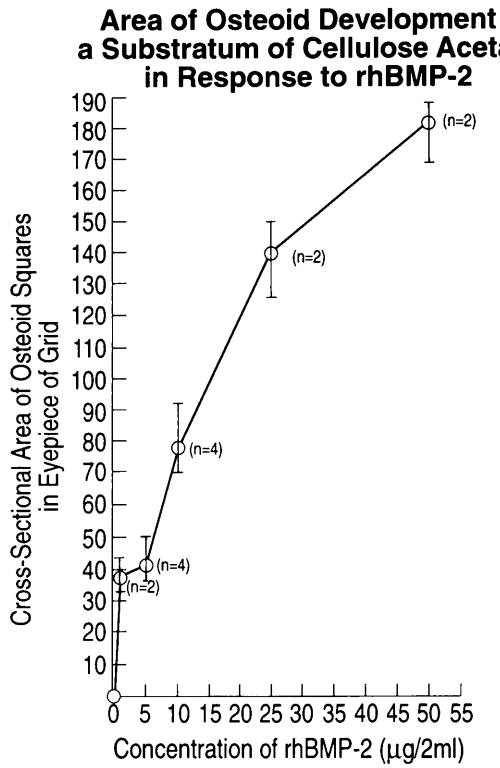

Figure 5. Graphic representation of relationship of area of osteoid to the quantity of rhBMP-2 supplements added with changes in the culture medium.

rhBMP-2 for 72 hr *in vitro,* and then transplanted *in vivo,* a radio-opaque area appeared on the outside of the chambers. Histological sections showed degenerated calcified muscle on the inside, and induced bone development on the outer membrane. With lower doses of rhBMP-2 and control chambers, without rhBMP-2, the chambers contained calcified degenerated muscle; fibrous tissue only developed on the outer membrane. These observations suggested that muscle derived connective tissue cell membranes either facilitate transmission or transduce a rhBMP-2 signal, or simply adsorb it.

Brain and Subcutis Derived Tissue. Outgrowths of connective tissue from brain onto cellulose acetate membranes, consisted of a loose mesenchymal type connective tissue. After 14–21 days of exposure to rhBMP-2, the outgrowths consisted of cartilage cells enclosed in a pale metachromatic staining matrix. Measured in terms of DNA-P, the quantity of cartilage was proportional to the dose of rhBMP-2.

Tendon and Periosteum Tissue Attachments. Control explants in three of seven cultures of periosteum and tendon attachments (without any rhBMP-2 supplements in the media) produced columns of hypertrophic cartilage. On the 21st day of cultivation, the explants consisted of cartilage mixed with islands of osteoid and woven bone. These explants were regarded as evidence that only the midsection of the muscle belly may be relied upon as a source of muscle-derived mesenchymal type cells.

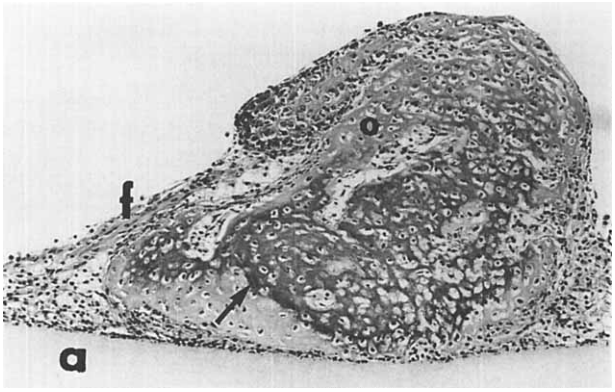

Figure 6. Photomicrograph of condroosteoid developed in outgrowth of muscle connective tissue on a substratum of cellulose acetate in cultured medium on Day 0, 1, and 2, supplemented with 50 μg of rhBMP-2, 14 days after explantation. Magnification: ×100.

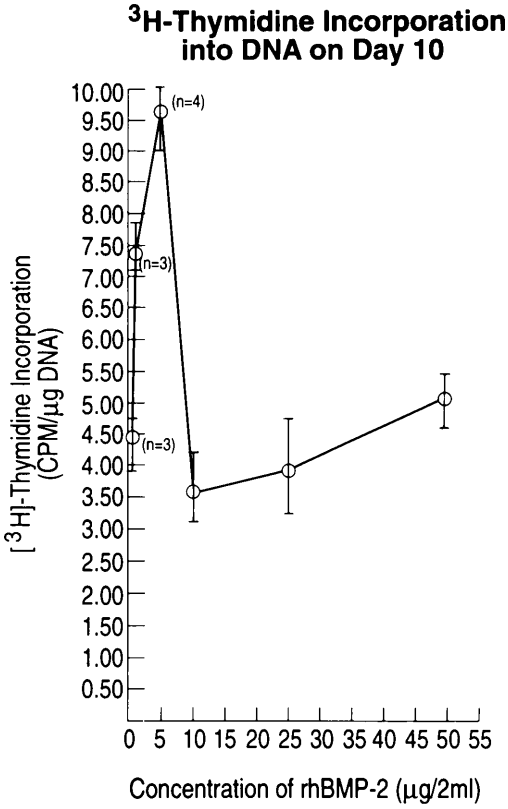

Figure 7. Graphic representation of the relationship of the incorporation of [^{3}H]thymidine into DNA and the concentration of rhBMP-2 (µg/ml) in the culture medium on Day 0, 1, and 3, and observed on the 10th day of cultivation.

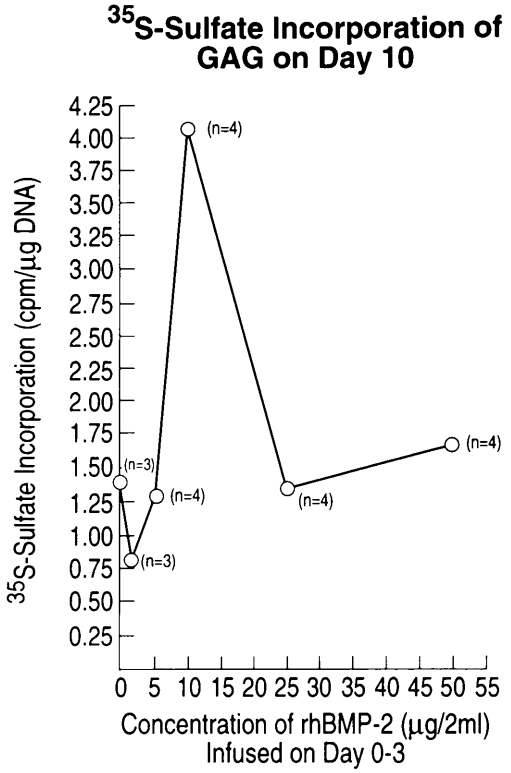

Figure 8. Graphic representation of the incorporation of [^{35}S]sulfate into glycosaminoglycans (GAG) in response to increasing concentrations of rhBMP-2 in the culture medium on Day 0, 1, and 3, and observed on the 10th day of cultivation.

Discussion

The foregoing observations present evidence that calcifiable bone can develop *de novo* under avascular conditions in tissue culture. The bone developed in connective tissue outgrowths of muscle without any intermediate enchondral phase of development. Under the influence of rhBMP-2 supplements of the culture medium, patches of intramemtranous bone formation differentiated from mesenchymal type cells. Exposure to rhBMP-2 for the first 3 days of cultivation was sufficient to induce differentiation of a calcifiable osteoid matrix 11 days later. The percentage of cultures with calcifying osteoid increased with increase in levels of rhBMP-2 in the range of 1–50 µg. With the native partially purified BMP added to the culture medica, we found cartilage only in muscle-derived connective tissue outgrowths (19, 20). Whether native partially purified hBMP consists of more than one or more than one molecular species is not known. rhBMP-2, like other products of recombinant DNA technology, was derived from amino acid sequences of tryptic peptides, not from undegraded native BMP.

Previous investigators cultured osteoprogenitor cells derived from mouse limb buds, chick periosteum, and fetal calvarial cells in order to cultivate new bone *in vitro* (29–32). β-Glycerol phosphate was added to the culture media for mineralization *in vitro* (29–32). With the CMRL-1066 culture medium, we found that β-glycerol phosphate supplements were not required to produce mineralization. In reviewing the ingredients of four standard culture media, we noted that the levels of inorganic phosphate, inorganic calcium, or ascorbic acid were higher in CMRL-1066 (Table II).

The levels of alkaline phosphatase activity were four to ten times above the controls and coincidental with the time of differentiation of osteoprogenitor cells. No one mechanism, either enzymic or nonenzymic, including alkaline phosphatase activity has been unequivocally proven to regulate mineralization of bone

Table II. Comparison of the Amounts of Inorganic Phosphate, Inorganic Calcium, and Ascorbic Acid in Various Media

Media type	Calcium (µg/ml)	Phosphate (µg/ml)	Ascorbic acid (µg/ml)
DMED	200	125	None (+50 µg/ml)[a]
$BGJ_β$	None	250	50 (+300 µg/ml)[a]
αMEM	200	140	50 (+50 µg/ml)[a]
CMRL-1066	2000	1400	500

[a] Supplementation.

(32). The enzyme has been correlated as often with phosphorylative glycogenolysis as with calcification.

BMP was serum dependent for optimum activity (33). Measured in terms of DNA and GAG synthesis, levels of BMP-induced cartilage development were low in serum free media. In serum supplemented media, 5 μg rhBMP-2 induced the optimum levels of DNA synthesis. Quantities greater than 5 μg appeared to inhibit DNA synthesis. Nanogram levels of BMP had only slight mitogenic effects on established cell lines of human skin, rat fetal skin, or bovine endothelium (23–27). The maximum synthesis of glycosaminoglycans (GAG) was associated with a 10 μg dose of rhBMP-2. In quantities of rhBMP-2, less than 10 μg, the changes in GAG synthesis were relatively small. Doses greater than 10 μg inhibited GAG production.

Synthesis of mature hyaline cartilage matrix occurred in response to partially purified native hBMP. In 5-μg supplements, rhBMP-2 induced membrane bone development without an intermediate endochondral stage, while native BMP induced development of cartilage only. Accumulation of hyaluronate within 24 hr and hyaluronidase within 48 hr was the earliest known marker for the morphogenetic phase of bone development. The cytodifferentiation phase of development had other biochemical markers (i.e., alkaline phosphatase, GAG, cartilage type proteoglycans, and α-carboxyglutamic acid–rich protein [BGP]) (25–27).

In diffusion chambers containing BMP, BMP-induced cells, or osteosarcoma cells on the inside, bone development occurred on the outside (15). With rhBMP-2 and muscle-derived mesenchymal cells on the inside, a favorable concentration gradient for bone development could form on the inside (29). When cells were exposed to rhBMP-2 first *in vitro* for 1–3 days and then transplanted into a diffusion chamber in the anterior abdominal wall of an autogeneic rat, the minimum effective dose of rhBMP-2 for transmembrane bone development was 0.2–5 μg. Based upon the response of bone marrow stroma cell lines C3H10T1-2 (23), as little as 100 ng induced cartilage and adipose cell differentiation. Other systems required 1 μg, considerably higher levels than reported for IGF, EGF, TGF, and various growth factors (23–27). Based upon the level of maximum alkaline phosphatase activity in W-20-17 stromal cells obtained from mouse bone marrow, Thies *et al.* (25) found that the minimum dose of rhBMP-2 was 6 ng/ml and the maximum dose was 800 ng/ml. Based on alkaline phosphatase activity of RBO-C26 (an osteoprogenitor cell line) the effective dose level was only 10 ng/ml (26). RBO-20, a mature osteoblast cell line responded similarly (33). RBO-C26 cells which differentiate into muscle cells and adipocytes *in vitro* were inhibited by rhBMP-2 (34). These observations suggested that the established cell lines that responded to nanogram doses of rhBMP-2 were predifferentiated with respect to cartilage and bone. Nonestablished nonautonomous cells, which normally do not form cartilage or bone in the lifetime of the individual, may require time for both quantal cell cycles and relatively larger doses of rhBMP-2.

All connective tissues of the body include cells that, like bone marrow stromal cells, may be competent (but not determined) to differentiate into bone in response to rhBMP-2. Rickard *et al.* (35) demonstrated that dexamethasone (10^{-8}) supplements in tissue culture media induce differentiation of marrow stroma cells into cartilage and bone. This response may be indicative of osteoprogenitor cells derived from bone marrow stroma. The effects of the dexamethasone and the BMP appeared to be additive. The measurements of alkaline phosphatase and other markers for bone cell differentiation suggested that the dexamethasone and rhBMP-2 have additive effects on marrow stroma cell lines. A pluripotential nonosteogenic mesenchymal progenitor cell line (C3H10T1/2) differentiated into osteoprogenitor cells, cartilage, osteoblasts, and adipose tissue in response to nanogram doses of rhBMP-2. These observations suggest that while bone marrow stroma cells respond to rhBMP-2, much large doses are required to induce differentiation of primary cell cultures of muscle-derived connective tissue cells into bone, either *in vivo* or *in vitro,* in the systems and conditions we and others employ at this time.

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1. Urist MR, Nogami H. Morphogenetic substratum for differentiation of cartilage in tissue culture. Nature **225**:1051–1052, 1970.
2. Nogami H, Urist MR. A substratum of bone matrix for differentiation of mesenchymal cells into chondro-osseus tissues in vitro. Exp Cell Res **63**:404–410, 1970.
3. Nogami H, Urist MR. Explants, transplants, and implants of a cartilage and bone morphogenetic matrix. Clin Orthop **103**:235–251, 1974.
4. Nogami H, Urist MR. Substrata prepared from bone matrix for chondrogenesis in tissue culture. J Cell Biol **62**:510–519, 1974.
5. Urist MR, Terashima Y, Stamos C. Cartilage tissue differentiation from mesenchymal cells derived from mature muscles in tissue culture. In Vitro **14**(8):697–706, 1978.
6. Nogami H, Oohira A, Terashima Y, Urist MR. Radioactive isotope labelled diffusible components of a bone morphogenetic protein. Clin Orthop **122**:307–314, 1977.
7. Nathanson MA. Transdifferentiation of skeletal muscle into cartilage: Transformation or differentiation? Curr Top Dev Biol **20**:39–62, 1986.
8. Nathanson MA. In vitro proteoglycan synthesis in response to extracts of demineralized bone. Clin Orthop **299**:263–281, 1994.
9. Nakagawa M, Urist MR. Chrondrogenesis in tissue cultures of

muscle under the influence of a diffusible component of bone matrix. Proc Soc Exp Biol Med **154**:568–572, 1977.
10. Sato K, Urist MR. Bone morphogenetic protein-induced cartilage development in tissue culture. Clin Orthop **183**:180–187, 1984.
11. Kataoka H, Urist MR. Transplant of bone marrow and muscle-derived connective tissue cultures in diffusion chambers for bioassay of bone morphogenetic protein. Clin Orthop **286**:262–270, 1974.
12. Terashima Y, Urist MR. BrdU-inhibition of substratum controlled chondrogenesis. Proc Soc Exp Biol Med **146**:855–858, 1974.
13. Terashima Y, Urist MR. Chondrogenesis in outgrowths of muscle tissue onto modified bone matrix in tissue culture. Clin Orthop **127**:248–255, 1977.
14. Urist MR, Iwata H. Preservation and biodegradation of the morphogenetic property of bone matrix. J Theor Biol **38**:155–167, 1973.
15. Urist MR, Granstein R, Nogami H, Svenson L, Murphy R. Transmembrane bone morphogenesis across multiple-walled chambers: New evidence of a diffusible bone morphogenetic property. AMA Arch Surg **112**:612–619, 1977.
16. Katoh R, Urist MR. Surface adhesion and attachment factors in bone morphogenetic protein-induced chondrogenesis in vitro. Clin Orthop **295**:295–304, 1993.
17. Kawamura M, Urist MR. Growth factors, mitogens, cytokines, and bone morphogenetic protein in induced chodrogenesis in tissue culture. Dev Biol **130**(2):435–442, 1988.
18. Murray SS, Murray EJB, Glackin CA, Urist MR. Bone morphogenetic protein inhibits differentiation and affects expression of helix-loop-helix regulatory molecules in myoblastic cells. J Cell Biochem **53**:1–10, 1993.
19. Hanamura H, Higuchi Y, Nakagawa M, Iwata H, Urist MR. Solubilized bone morphogenetic protein (BMP) from mouse osteosarcoma and rat demineralized bone matrix. Clin Orthop **148**:281–290, 1980.
20. Takahashi S, Urist MR. Differentiation of cartilage in three substrata under the influence of an aggregate of morphogenetic protein and other bone tissue non collagenous proteins (BMP/iNCP). Clin Orthop **207**:227–238, 1986.
21. Urist MR. The search for and discovery of bone morphogenetic protein (BMP). In: Urist MR, Burwell RG, O'Conner BT, Eds. Bone Grafts, Derivatives and Substitutes. London: Butterworth Heinemann, pp315–362, 1994.
22. Reddi AH. Regulation of cartilage and bone differentiation by bone morphogenetic proteins. Curr Opin Cell Biol **4**(5):850–855, 1992.
23. Wang EA, Israel DI, Kelly S, Luxenburg DP. Bone morphogenetic protein 2 commitment and differentiation in $C_3H1OT1/2$ and 3T3 cells. Growth Factors **9**:57–71, 1993.
24. Israel DI, Nove J, Kerns KM, Moutsatsos IK, Kaufman RJ. Expression and characterization of bone morphogenetic protein-2 in Chinese hamsters ovary cells. Growth Factors **7**:139–150, 1992.
25. Thies RS, Bauduy M, Ashton BA, Kurtzberg L, Wozney JM, Rosen V. Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stroma cells. Endocrinology **130**:1318–1324, 1992.
26. Katagiri T, Yamaguchi A, Ikeda T, Yoshiki S, Wozney J, Rosen V, Wang FA, Tanaka H, Omura S, Suda T. The non osteogenic mouse pluripotent cell line, $C_3H1OT1/2$ is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Commun **172**:295–299, 1990.
27. Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, Wang FA, Kahn AJ, Suda T, Yoshiki S. Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. J Cell Biol **113**(3):681–687.
28. Healy GM, Parker RC. An improved chemically defined basal medium (CMRL). J Cell Biol **30**:531–538, 1966.
29. Urist MR. Heterotopic bone formation. In: Urist MR, Ed. Fundamental and Clinical Bone Physiology. Philadelphia: J. B. Lippincott, pp369–393, 1980.
30. Tenebaum HD, Heersche JNM. Differentiation of osteoblasts and formation of mineralized bone in vitro. Calcif Tissue Int **34**:76–79, 1982.
31. Tenebaum HD, Limeback H, McCulloch CAG, Mamujee H, Sukhu B, Torontali M. Osteogenic phase-specific co-regulation of collagen synthesis and mineralization by β-glycerophosphatase in chick periosteal cultures. Bone **13**:129–138, 1992.
32. Firschein HE, Urist MR. The induction of alkaline phosphatase by extraskeletal implants of bone matrix. Calcif Tissue Res **7**:108–113, 1971.
33. Bellows CG, Aubin JE, Heersche JNM, Antosz ME. Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations. Calcif Tissue Int **38**:143–154, 1986.
34. Zimmermann B, Wachtel JC, Vormann J. Kinetics of β-glycerophosphatase–induced endochondral mineralization in vitro. Calcium accumulation, alkaline phosphatase activity, and effects of levamisole. Calcif Tissue Int **51**:54–61, 1992.
35. Rickard DJ, Sullivan TA, Shenker BJ, LeBoy PS, Cazhdan HI. Induction of rapid osteoblast differentiation of rat bone marrow stromal cell cultures by dexamathasone and BMP-2. Dev Biol **161**:218–228, 1994.