PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 173, 194–199 (1983)

Human Bone Morphogenetic Protein (hBMP)[1] (41630)

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Abstract. Human bone morphogenetic protein (hBMP) was chemically extracted from demineralized gelatinized cortical bone matrix by means of a $CaCl_2$ · urea inorganic–organic solvent mixture, differential precipitation in guanidine hydrochloride, and preparative gel electrophoresis. hBMP is isolated in quantities of 1 mg/kg of wet weight of fresh bone, and has the amino-acid composition of an acidic polypeptide. The mol wt is 17 to 18 k-Da (kilodaltons). Implants of the isolated 17-kDa protein are very rapidly adsorbed and produce a smaller volume of bone than protein fractions consisting of 24-, 17-, and 14-kDa proteins. Since the isolated 24- and 14-kDA components lack hBMP activity, the kinetics of the bone morphogenetic processes including the function of other proteins as carrier molecules, await investigation.

Cartilage and bone cell differentiation is induced in postfetal life in mammalian species under the influence of a bone morphogenetic protein (BMP) (1). Implants of BMP in muscle pouches, diffusion chambers, and subcutaneous spaces consistently induce perivascular mesenchymal-type connective tissue cells to differentiate into cartilage by 7 days, woven bone by 14 days, and lamellar bone including bone marrow by 21 days. Crude and partially purified BMP has been extracted from rabbit dentin (2), bovine cortical bone (3), mouse (4–6) and human osteosarcoma tissue (7) with the aid of collagenase (8), ethylene glycol (9), calcium chloride urea (3), and GuHCl solution (10). We report here on special measures required for extraction of BMP from human bone (hBMP) with a demonstration of its association with other water-insoluble low-molecular-weight bone matrix proteins and some individual biochemical characteristics.

Materials and Methods. hBMP was extracted from 10 kg of femoral and tibial cortical bone obtained (within 12 hr after death) from randomly selected, male autopsy subjects, 29 to 52 years of age. The bone was frozen in liquid N_2, pulverized, defatted, demineralized, converted into 1.2 kg of bone-matrix gelatin, and freeze-dried by methods described in a previous communication (8). The hBMP was isolated by the procedures shown in the flow diagram in Fig. 1.

The initial $CaCl_2$ · urea-soluble, water-insoluble, protein fraction was assayed for hBMP activity by implantation in muscle pouches in the hindquarters of Swiss Webster mice. A dose–response curve was obtained from implants weighing 0.5, 1.0, 2.0, and 3.0 mg, by measurements of ^{45}Ca uptake by the calcifying new bone deposits per milligram of implanted protein. For control measurements, unimplanted and control implanted muscle tissue of the contralateral thigh of comparable weight was excised and processed in exactly the same method as the muscle implanted with BMP.

hBMP was purified by a combination of Sepharose CL-6B gel filtration and preparative gel electrophoresis. The individual fractions were dialyzed against cold water to remove GuHCl and to precipitate the water-insoluble proteins. Lyophilized weighed samples of each fraction were incubated for 24 hr in 0.1 *M* sodium phosphate buffer containing 2 *M* urea and 0.1% sodium dodecyl sulfate (SDS), pH 7.2, for polyacrylamide gel electrophoresis (PAGE); 5 μl (2.5 mg/ml) samples were applied to 8.5% gel, electrophoresed at

[1] These investigations were supported by grants-in-aid from USPHS, NIDR (DEO2103), The Kroc Foundation, and the Solo Cup Foundation.

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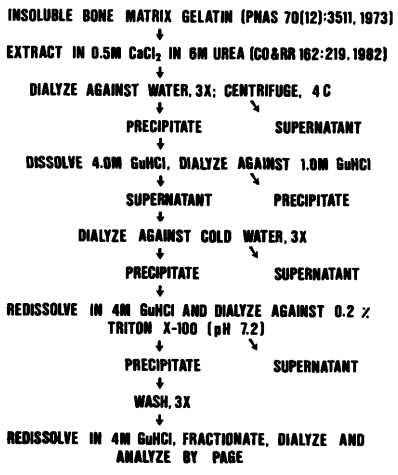

FIG. 1. Flow diagram of a method of chemical extraction of hBMP from cortical bone.

25 mA, and stained with 0.044% Coomassie brilliant blue R-250.

For preparative tube gel electrophoresis and amino acid analysis of individual proteins, 6-mm slabs and 1-cm tubes of 15% PA were loaded with a 0.2% SDS and 0.12% urea solution of hBMP; 1.7 μg/tube was run at 15 mA/tube for 18 hr. Gels were sliced with a knife across zones corresponding to 24-, 17-, and 14-kDa proteins using duplicates stained for orientation. Gel slices containing the 17-kDa hBMP were extracted with 0.2% SDS, dialyzed against water, and lyophilized; 200-μg samples were hydrolyzed under vacuum for 24 hr at 110°C in 1.0 ml of 6 *N* HCl, and applied to an amino-acid analyzer (equipped with a Spectrum Physics data reduction system). Similarly prepared 24- and 14-kDa protein fractions were analyzed for comparison.

Results. The dose–response curve to implants of hBMP, prepared by the procedure shown in Fig. 1, is summarized in Fig. 2. The incorporation of ^{45}Ca into histologically valid deposits of new bone was directly proportional to the weight of implanted hBMP, ranging from 22 to 102 cpm $^{45}Ca \times 10^{-3}$/ml. In contrast, the unimplanted contralateral muscle emitted only 3 cpm. The correlation coefficient of the hBMP to induced bone formation was 0.9924. Control implants of human tendon collagen and serum albumin invariably failed to induce bone formation. Implants of the isolated 17-kDa (kilodalton) fraction in microgram quantities were absorbed in less than 24 hr, without producing detectable quantities of new bone. Implants of predominately 24- and 14-kDa protein fractions in 10-mg quantities did not induce bone formation.

The fractionation of the extracts obtained by Sepharose CL-6B gel filtration and preparative tube gel electrophoresis is shown in Fig. 3A–C. Bone morphogenetic activity was correlated with the presence of the 17-kDa component separated in fraction IV (Fig. 4). Twenty implants of the original extract, 4 of fraction IV (consisting of the 17-kDa and associated 24- and 14-kDa components) invariably produced deposits of new bone, whereas 108 implants (collected from three separate batches of bone) lacking the 17-kDa component failed to induce bone formation. Mercaptoethanol reduction extinguished BMP activity without perceptively altering the electrophoretic pattern of either fraction IV or the 17-kDa protein.

The amino acid composition of the 17-kDa protein is shown in Table I. Serine, aspartic acid, glutamic acid, and glycine were the predominant amino acids. The mol wt calculation, based upon the sum of the individual weights of amino acids (not including tryptophan) was approximately 17,684. The total carbohydrate contents were approximately

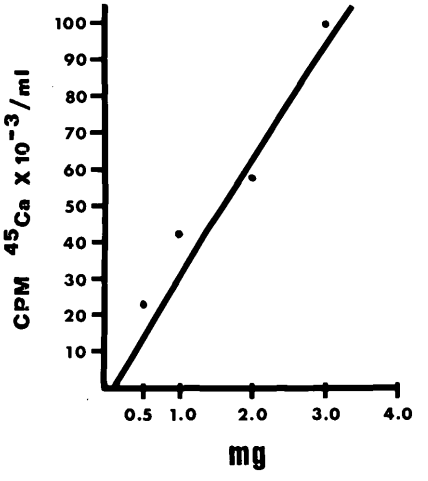

FIG. 2. hBMP dose response. Graphic demonstration of the yield of calcified new bone in direct proportion to the quantity of hBMP implanted in muscle in the mouse thigh.

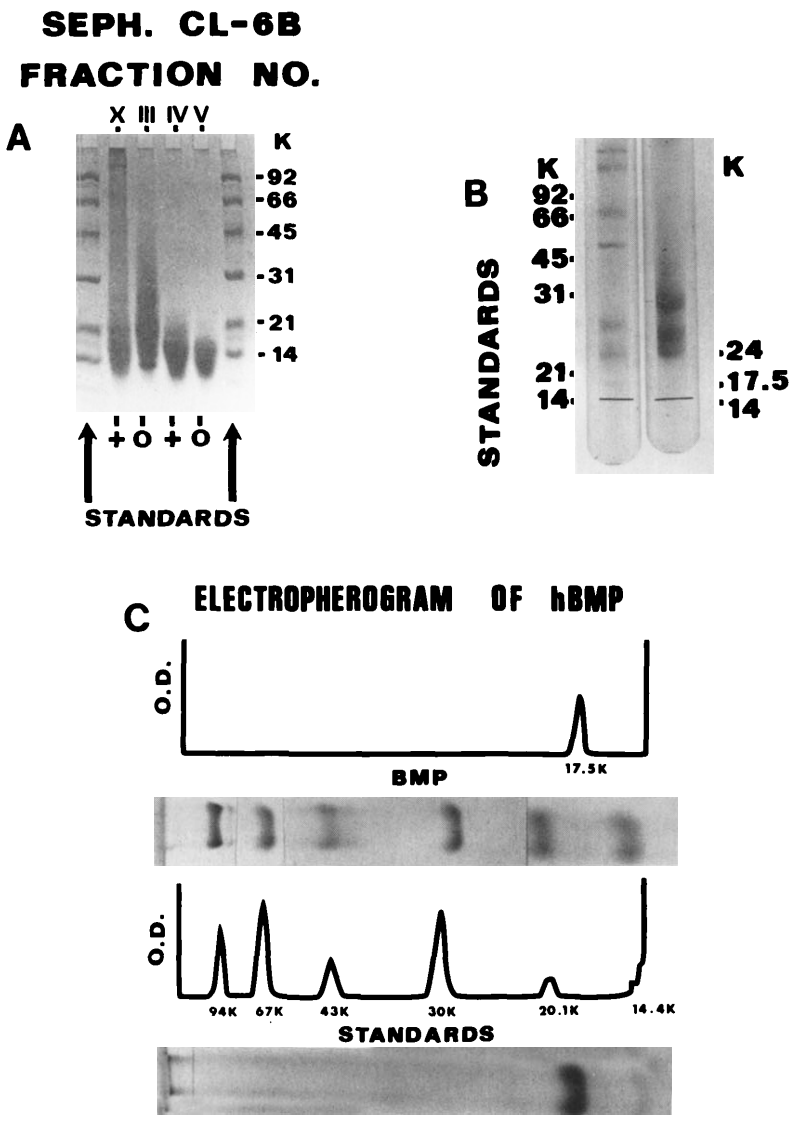

FIG. 3. hBMP. (A) Slab gel electrophoresis patterns of the major fractions obtained by gel filtration. Fraction X is the starting material. The biologically active fraction IV is labeled (+) and the inactive fractions III and V are labeled (0). The assigned mol wt is designated in kilodaltons (kDa). (B) Tube gel electrophoresis pattern of the biologically active fraction IV (CL-6B) showing three Coomassie blue-staining components. (C) Electropherogram and disc gel electropherogram of an isolated 17.5-kDa component.

12% of the CL-6B fraction IV with none detectable in the 17-kDa component.

Discussion. As described above, hBMP has the biochemical characteristics of a relatively insoluble polypeptide that is closely associated with three other equally insoluble low-molecular-weight, electrophoretically defined proteins. The 34-kDa component is a Triton X-100-soluble, water-soluble glycoprotein that is relatively easy to separate in large quantities. The 24- and 14-kDa components are difficult to separate from each other and from the 17-kDa polypeptide by differential precipitation, gel filtration, and preparative gel electrophoresis.

The 17-kDa protein is invariably present in chromatographic fractions having high hBMP activity, generally absent in protein fractions

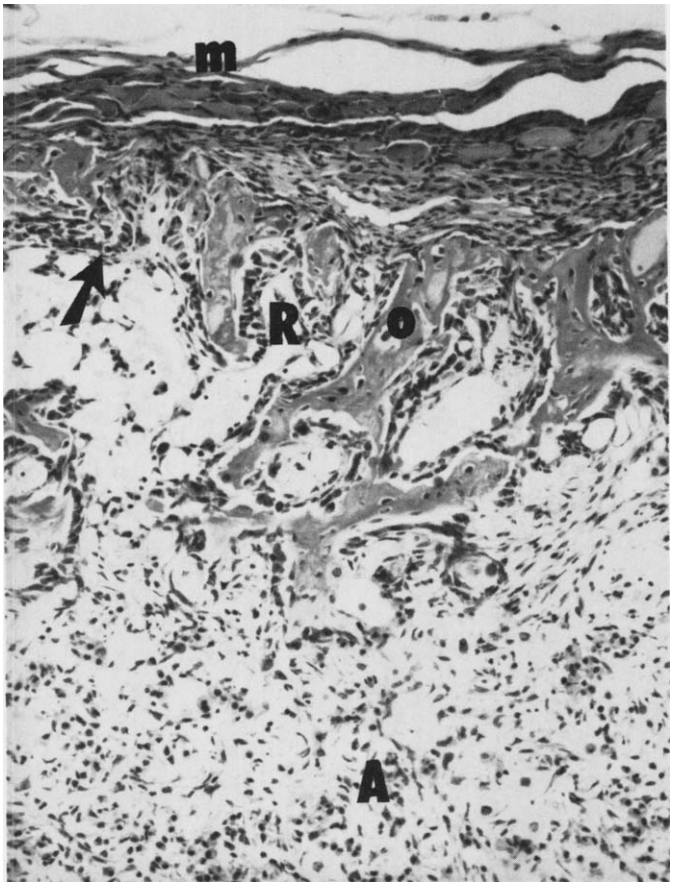

FIG. 4. Photomicrograph of deposit of new bone formed in muscle in response to an implant of 2.0 mg of hBMP. Note: muscle (m), bone (o), marrow (R), mesenchymal type tissue (A), osteoblasts (arrow).

lacking hBMP activity, and is therefore the putative hBMP. More important, the isolated 34-, 24-, and 14-kDa components do not induce bone formation. Amino-acid analyses suggest that hBMP is an acidic polypeptide similar in composition to a previously reported bovine BMP (10).

Relative to the microgram and picogram doses of hormones, vitamins, and metabolites that produce measurable biological reactions, the milligram doses of hBMP required to induce bone formation *in vivo* are extraordinarily high. Implants of about 1.0 mg of the isolated hBMP induce formation of hardly detectable volumes of bone. Since 1.0 mg of protein fractions including 24- and 14-kDa proteins induce formation of a relatively large volume of new bone, it is reasonable to assume that the impurities may serve as carriers. However, 5 to 10 mg of either the impure or purified hBMP is required to induce grossly visible bone formation, and the yield is proportional to the mass of implanted protein. Small quantities of hBMP are either too rapidly absorbed or too completely degraded by tissue proteases, i.e., BMPases (11), for a sufficient number of responding mesenchymal cell populations to develop.

The biochemical interactions and target cell mediated mechanisms of recognition of BMP are not known. Information is also insufficient on whether in the intact bone matrix 17-kDa accompanying 24- and 14-kDa components are macromolecular subunits produced only under dissociative conditions in 4 *M* GuHCl or 6 *M* urea. A previously published observation that mercaptoethanol reduction eliminates and reoxidation restores

TABLE I. AMINO ACID ANALYSIS OF HUMAN BMP ISOLATED BY SDS EXTRACTION OF PAGE SLICES

Amino acids	Nanomoles*	Mole %	Residue / 17,000	Weight / Mole protein
Lys	2.05	4.09	7.16 (7)	897.19
His	0.83	1.66	2.91 (3)	411.42
Arg	3.51	7.00	12.25 (12)	1874.16
Asx	4.00	7.98	13.97 (14)	1611.12
Thr	1.54	3.07	5.37 (5)	505.50
Ser*	4.03	8.04	14.07 (14)	1218.98
Glx	5.10	10.17	17.80 (18)	2323.98
Pro	1.86	3.71	6.49 (6–7)	582.66
Gly	12.45	24.83	43.45 (43)	2453.15
Ala	3.40	6.78	11.87 (12)	852.84
1/2Cys*	0.98	1.95	3.41 (3–4)	309.42
Val*	2.20	4.39	7.68 (8)	793.04
Met	0.48	0.96	1.68 (2)	262.38
Ile*	1.55	3.09	5.41 (6)	678.90
Leu	3.40	6.78	11.87 (12)	1357.80
Tyr	1.45	2.89	5.06 (5)	815.85
Phe	1.31	2.61	4.57 (5)	735.85
	50.14	100.00	175–177 + Trp + $(CH_2O)\times$	17,684 + Trp + $(CH_2O)\times$

* Nanomoles for each amino acid = average of 24- and 72-hr hydrolysates or the extrapolated value (Ser) or the 72-hr value only (Val and Ile) or the value from the hydrolysate of the performic acid-oxidized sample (1/2Cys).

osteoinductive activity of whole-bone matrix is evidence that the BMP component is a disulfide-bonded molecule (12). Reduction of the 17-kDa and associated 24- and 14-kDa molecules did not appreciably change the electrophoretic pattern, indicating that disulfide bonds may be present very near one or the other end of the BMP molecule.

BMP differs from bone-derived growth factors (BDGF) identified by Canalis *et al.* (13) and Farley *et al.* (14) in both biochemical and biologic properties. BMP is relatively insoluble, whereas BDGF is soluble in aqueous media. BDGF stimulates DNA synthesis to peak levels within hours whereas BMP requires 3 to 4 days (15). The BDGF target is a predifferentiated cartilage or osteoprogenitor cell line. The BMP target is a perivascular mesenchymal-type pericyte. BMP induces cell disaggregation, migration, and reaggregation of an entirely new cell population before it induces differentiation of cartilage and bone (1). The process is irreversible and culminates in the development of an ossicle which then remains for the lifetime of the organism. In contrast, the process of bone cell-growth stimulation by BDGF is reversible.

BMP induces interactions of highly complex extra- and intracellular processes leading to generation of an entire embryonic type bone morphogenetic program. Consequently, either indirectly or directly, BMP must activate DNA sequences that code for bone morphogenesis. In response to BMP, some restriction enzymes affected by DNA methylation could turn off gene expression, whereas unaffected DNA sequences would turn them on for bone morphogenesis; the methylation pattern is heritable (16, 17). Conceptually, BDGF is a paracrine–autocrine agent (18) that releases the constraints upon DNA activity whereas BMP is a morphogen (1) that initiates a new DNA sequence.

BMP is believed to be the biochemical constituent of demineralized human bone recently found to stimulate regeneration of craniofacial bone defects caused by injury, malignancy, infection, and congenital deformity (19–22). Although the quantity of BMP in transplants of bone matrix is very small, methods are available to produce quantities sufficient for preliminary clinical applications. Almost unlimited quantities of BMP could be purified by antibody-affinity chromatography or by recombinant-DNA technology. A highly purified BMP would be useful for both fundamental and clinical research on the remarkable capacity of bone for con-

tinuous cell differentiation, regeneration, and internal remodeling throughout postfetal life.

The authors are indebted to Vincent Hascall and associates for the carbohydrate analyses, and to Paul Price for helpful suggestions and assistance.

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Received September 8, 1982. P.S.E.B.M. 1983, Vol. 173.