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Radioimmunoassay of Bone Morphogenetic Protein in Serum: A Tissue-Specific Parameter of Bone Metabolism[1] (41900)

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Abstract. Bone morphogenetic protein (BMP), a paracrine agent inducing cartilage and bone cell differentiation, circulates in the blood and is detectable by BMP radioimmunoassay. Serum BMP levels are higher in growing children and patients with Paget's disease than in normal adults. These observations are interpreted as evidence of a BMP function in the physiology of bone in health and disease.

Bone is unique among the connective tissues of the body in several respects. First, it is continuously remodeled irrespective of physiological demands and aging. Second, in response to injury it regenerates without scar, completely and flawlessly. Third, bone develops *de novo* in extraskeletal sites by differentiation of perivascular connective tissue cells (pericytes) under the influence of a bone morphogenetic protein (BMP) derived from bone (1–4), dentin (5), and osteosarcoma tissues (6, 7). Bovine BMP (bBMP) is an acidic polypeptide with a relative molecular mass (M_r) of 18,500 ± 1000 and pI of 5.0 ± .1 (4). Human BMP (hBMP) has similar characteristics with a slightly lower M_r. This report is submitted as evidence that BMP circulates in the blood in nanogram quantities, detectable by a standard displacement method of radioimmunoassay (RIA).

Materials and Methods. *Preparation of antigen.* bBMP was prepared from bovine cortical bone and purified by methods described in a recent report (4).

Preparation of antibodies. An antibody was prepared by injection of purified bBMP into rabbits, 5 mg/kg in Freund's adjuvant, 5 mg/ml sc/week for 3 weeks. The serum was collected 3 days after the last injection.

Iodination of BMP. BMP was iodinated using a modification of the method of Bolton and Hunter (8). To 10 μg of BMP in 50 μl phosphate-buffered saline (PBS), pH 7.0, were added 4 μCi of ^{125}I (Amersham) and 10 μl of chloramine T (5 mg/12.5 ml). The reaction was allowed to continue for 180 sec and terminated with the addition of 10 μl sodium metabisulfite (5 mg/10 ml). This product was then purified on a 0.7 × 25-cm column of Sephacryl S-200 (Pharmacia, Piscataway, N.J.) using 0.1 *M* PBS, pH 6.55, containing 0.05% Tween 20.

Immunoassay. Duplicate samples of serum were analyzed both quantitatively and qualitatively by enzyme-linked immunosorbent assay (ELISA), using the dot immunobinding method (9).

Serum analyses. Thirty adult human serum samples (0.5 ml) were collected from the discarded surplus stock of blood analyzed by the hospital clinical chemistry laboratory, and classified as normal. Sera from six different children similarly classified, were obtained from the tissue typing laboratory. Serum from three patients with severe Paget's disease, two male and one female, (with very high levels of serum alkaline phosphatase levels) were obtained from the surplus of blood collected for routine diagnostic studies.

Controls. The three following control analyses were performed on each sample to exclude unspecific protein reactions. (a) Human serum stripped of protein hormones with dextran-coated charcoal (NORIT-SG, Fischer Chem, Los Angeles, Calif.), (b) serum supplemented with 6 to 16 ng of prolactin, (c) serum supersaturated with 100 ng of prolactin.

Results. Figure 1 shows a dose–response curve of the RIA for BMP when plotted on a log–log scale. The concentration of BMP is plotted on the horizontal axis, while the per-

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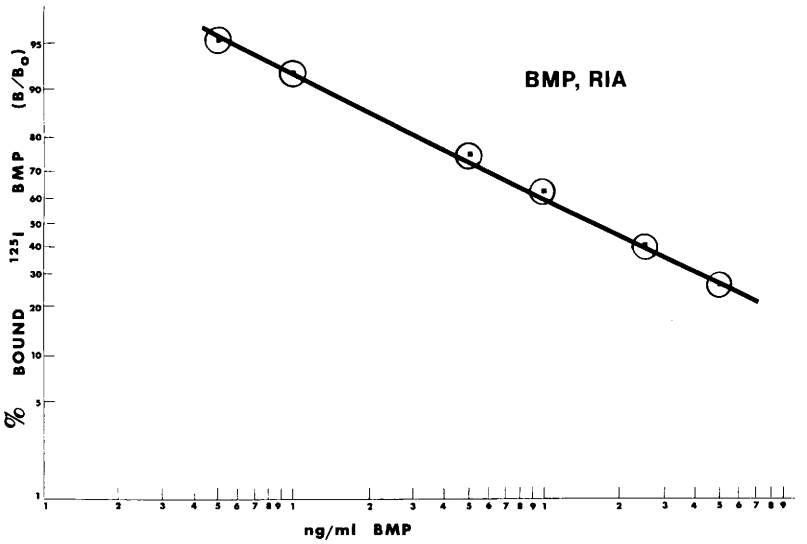

FIG. 1. A standard linear dose–response relationship (log–log scale) showing the sensitivity of the BMP RIA with ^{125}I-BMP binding to rabbit anti-bBMP antibody in ng range of values.

centage bound BMP ^{125}I is plotted on the vertical axis as the ratio of B/Bo. "Bo" represents the maximum quantity of ^{125}I-BMP bound to the antibody in the absence of nonradioactive BMP. "B" represents the amount of ^{125}I-BMP bound to the antibody when known concentrations of nonradioactive BMP are present. In order to obtain maximum displacement, a 200-μl sample size was used.

To validate an RIA, one must show that the antigen used for the standards is immunologically identical to the material being measured in the serum (10–12). Therefore, we use the parallelism and recovery methods.

Table I demonstrates the parallelism or effects of dilutions on the assay. Serial dilutions of two samples with a high concentration of BMP were made from 0- to 16-fold. If both standard and serum BMP are immunologically identical then the values obtained from a diluted sample multiplied by its dilution factor should be within experimental error, identical to the undiluted value. This is essentially the case.

TABLE I. PARALLELISM OF BMP RIA[a]

Dilution		Expt A[b]	Expt B[c]
0	Value obtained	370	160
1:2	Value obtained	180	84
	Value (×) dilution factor	180 × 2 = 360	84 × 2 = 168
1:4	Value obtained	80	38
	Value (×) dilution factor	80 × 4 = 320	38 × 4 = 152
1:8	Value obtained	40	19
	Value (×) dilution factor	40 × 8 = 320	19 × 18 = 152
1:16	Value obtained	20	11
	Value (×) dilution factor	20 × 16 = 320	11 × 16 = 176

[a] All values are ng/ml BMP.
[b] Expt A: mean, 338.0; SD, 24.9.
[c] Expt B: mean, 161.6; SD, 10.4.

Table II further demonstrates the accuracy of this BMP RIA by its capacity to detect the additive BMP concentrations. In Experiment A with initial (baseline) value of 17 ng/ml, and Experiment B with 50 ng/ml, the total RIA values prove to be acceptably close to the sum of the initial values plus the added quantities of BMP, showing that the BMP used for the standards and the serum BMP must be immunologically identical.

The following experiment was set up to test the precision or reproducibility of the assay. Three samples were analyzed, followed by the standard curve, and again followed by three samples. When the system reached equilibrium, the samples were read and the same value obtained, regardless of the position in the assay. Thus it was possible to eliminate any question arising from the length of the assay or the number of samples being analyzed. The experiment was also set up on five different days, and on each occasion the equilibrium was complete. Table III shows average values obtained per assay run, and good *inter-* and intraassay correlation.

Table IV summarizes the results obtained by performing an RIA on the serum of 94 patients including rapidly growing normal children, normal adults, and three patients with Paget's disease. The range of BMP RIA values of normal adults was significantly lower than in growing children and about 17 times lower than three patients with extensive Paget's disease of the skull, vertebra, pelvis, femur, and tibia.

ELISA. With the ELISA procedure, the mouse anti-human BMP cross-reacted 100% with both human and bovine BMP. Similarly, the rabbit anti-bovine BMP cross-reacted 100% with both the human and bovine BMP which demonstrates that either BMP is not species specific, or the segment of the molecule our antisera detects may be common to several species. On samples listed in Tables I to III, with BMP RIA values greater than 15 ng/ml, the results were confirmed by an unequivocally positive qualitative ELISA.

TABLE II. RECOVERY OF BMP RIA

BMP added (ng)	Test A (17 ng/ml)		Test B (50 ng/ml)	
	Expected	Recovered	Expected	Recovered
250	267	280	300	320
125	142	150	175	190
50	67	64	100	90
25	42	38	67	72

Note. All values are ng/ml BMP.

TABLE III. BMP RIA DETERMINATIONS OF THREE SETS OF SAMPLES ON FIVE SUCCESSIVE DAYS

	BMP (ng/ml)		
	Serum A	Serum B	Serum C
	10	21	17
	12	24	19
	11	25	20
	14	27	17
	10	25	16
Mean	11.4	24.4	17.8
SD	1.67	2.19	1.64
Coeff. variation (%)	14.6	9.0	9.2

Discussion. The hypothesis supported by the above recorded data is that BMP is a normal byproduct of local or paracrine (3, 13) bone metabolism, that appears in the sera of normal individuals. BMP RIA values are higher in rapidly growing children with normally high levels of bone turnover than in nongrowing adults with normally low rates of bone turnover. Rapidly growing children have a range of at least 20 to 72 ng/ml, while normal adults show less than 16 ng. The standard deviation of the values for children was 18.9, compared with 5.7 and 4.40 for men and women, respectively, and was attributable to

TABLE IV. SERUM BMP IN HEALTH AND DISEASE

Conditions	No. patients	No. analyses	RIA (ng/ml)	
			Range	Mean
Adult men	35	69	1–23	14.43[a]
Adult women	45	100	13–35	18.20[b]
Children, ages 2 to 8	6	9	20–72	56.0[c]
Paget's disease	3	5	>250	ND

[a] SD 5.77.
[b] SD 4.40.
[c] SD 18.90.

one child with a value of 20 ng/ml compared to 5 others with values ranging from 54 to 72 ng/ml. A larger sample of normal and abnormal children and further improvements in the sensitivity of the assay are presently under investigation.

Three patients with extensive Paget's disease consistently show high levels, over 250 ng, of serum BMP. To investigate serum BMP RIA values in other disorders in which the rate of bone turnover is abnormal, more particularly the resorption phase, the present research is being extended to patients with osteoporosis, hyperparathyroidism, and renal osteodystrophy. Our observations on mice, rats, rabbits, and dogs with implants of BMP in muscle or in bone defects (1, 5) or BMP in mouse or human osteosarcomas (6, 7, 14, 15) and other bone tumors (16, 17) suggest that a BMP quantitative immunoassay could prove to be of value for diagnosis and the response to treatment.

The origin of serum BMP from tissues other than bone has a bearing on the interpretation of BMP RIA. We have assayed crude 4 *M* guanidinium HCl extracts of brain, liver, muscle, and kidney and found no detectable BMP activity. The possibilities of secretion of BMP by transitional epithelium of kidney pelvis, ureter, urinary bladder, and mucosa cells of stomach and gall bladder, nonosseous tissues known to induce bone formation (18), have not been entirely excluded. However, the total body skeletal tissue mass is so much greater than any other organ system that an extraosseous source would be more significant under pathological than normal physiological conditions.

1. Urist MR. Bone morphodifferentiation and tumorigenesis. Perspect Biol Med **22**:S89–S113, 1979.
2. Mizutani H, Urist MR. The nature of bone morphogenetic protein (BMP) fractions derived from bovine bone matrix gelatin. Clin Orthop **171**:213–223, 1982.
3. Urist MR, DeLange RJ, Finerman GAM. Bone cell differentiation and growth factors: Induced activity of chondro-osteogenetic DNA. Science **220**:680–686, 1983.
4. Urist MR, Huo YK, Brownell AG, Hohl WM, Buyske J, Lietze A, Tempst P, Hunkapillar M, DeLange RJ. Purification of bovine bone morphogenetic protein by hydroxyapatite chromatography. Proc Natl Acad Sci USA **81**:371–375, 1984.
5. Conover MA, Urist MR. Dentin matrix morphogenetic protein. In: Proceedings of the 1st International Conference on Chemistry and Biology of Mineralized Connective Tissue, Northwestern University, May 3–7, 1981. New York, Elsevier/North-Holland, pp597–606, 1982.
6. Takaoka K, Yoshikawa H, Shimizu N, Ono K, Amitani K, Nakata Y, Sakamoto Y. Purification of a bone-inducing substance (osteogenic factor) from a murine osteosarcoma. Biomed Res **2**(5):466–471, 1981.
7. Bauer FCH, Urist MR. Human osteosarcoma-derived soluble bone morphogenetic protein. Clin Orthop **154**:234–248, 1981.
8. Hunter WM, Greenwood FC. Preparation of Iodine 131 labelled human growth hormone of high specific activity. Nature (London) **194**:495–496, 1962.
9. Hawkes R, Niday E, Gordon J. A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem **119**:142–147, 1982.
10. Midgley AR Jr, Niswender, GD, Rebar RW. Principles for the assesment of the reliability of radioimmunoassay methods (precision, accuracy, sensitivity, specificity). Acta Endocrinol **63**(Suppl):142, 163.
11. Skelley DS, Brown LP, Besch PK. Radioimmunoassay. Clin Chem **19**:146–186, 1973.
12. Berson SA, Yalow RS, eds. In: Peptide Hormones. Amsterdam, North-Holland, p84, 1973.
13. Sporn MB, Todaro FJ. Autocrine secretion and malignant transformation of cells. N Engl J Med **303**:878–880, 1980.
14. Urist MR, Grant TT, Lindholm ST, Mirra JM, Hirano H, Finerman GAM. Induction of new-bone formation in the host bed by human bone-tumor transplants in athymic nude mice. J Bone Joint Surg **61A**(8):1207–1216, 1979.
15. Hanamura H, Higuchi Y, Nakagawa M, Iwata H, Nogami H, Urist MR. Solubilized bone morphogenetic protein (BMP) from mouse osteosarcoma and rat demineralized bone matrix. Clin Orthop **148**:281–289, 1980.
16. Bauer FCH, Urist MR. The reaction of giant cell tumors of bone to transplantation into athymic nude mice: Including observations on osteo-induction in the host bed. Clin Orthop **159**:257–264, 1981.
17. Bauer FCH, Mirra MJ, Urist MR. Bone induction by Ewing's sarcoma: Transplantation into athymic nude mice. Arch Pathol Lab Med **105**(6):322–324, 1981.
18. Huggins CB. The formation of bone under the influence of the epithelium of the urinary tract. Arch Surg **22**:337–389, 1931.

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