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Research Article

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New Biochemical Marker for Bone Metabolism

MEASUREMENT BY RADIOIMMUNOASSAY OF BONE GLA PROTEIN IN THE PLASMA OF NORMAL SUBJECTS AND PATIENTS WITH BONE DISEASE

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ABSTRACT γ-Carboxyglutamic acid-containing protein of bone (BGP) is an abundant noncollagenous protein of mammalian bone. BGP has a molecular weight of 5,800 and contains three residues of the vitamin K-dependent amino acid, γ-carboxyglutamic acid. We have applied a radioimmunoassay based on calf BGP for the measurement of the protein in the plasma of 109 normal humans and 112 patients with various bone diseases. BGP in human plasma was demonstrated to be indistinguishable from calf BGP by assay dilution studies and gel permeation chromatography. The mean (±SE) concentration of BGP in normal subjects was 6.78 (±0.20) ng/ml, 7.89 (±0.32) for males and 4.85 (±0.35) for females. Plasma BGP was increased in patients with Paget's disease of bone, bone metastases, primary hyperparathyroidism, renal osteodystrophy, and osteopenia. Plasma BGP did correlate with plasma alkaline phosphatase (AP) in some instances, but there were dissociations between the two. It was additionally observed that patients with liver disease had normal plasma BGP despite increased plasma AP, a reflection of the lack of specificity of AP measurements for bone disease. Our studies indicate that the radioimmunoassay of plasma BGP can be a useful and specific procedure for evaluating the patient with bone disease.

INTRODUCTION

The assessment of bone metabolism and bone disease remains a difficult problem in clinical medicine. The measurements of alkaline phosphatase in blood and hydroxyproline in urine have been the most widely used biochemical tests for this purpose (1, 2). Although these measurements are of considerable clinical value, each has its limitations. Alkaline phosphatase is not a specific reflection of bone function, because its concentration in blood is also contributed to by the liver, gastrointestinal tract, placenta, certain tumors, and perhaps other sources (1). Hydroxyproline is similarly not specific for bone because it can be influenced by diet and nonosseous as well as osseous collagen (2).

We have applied to clinical studies of bone metabolism an assay for the vitamin K-dependent protein of bone. This protein was discovered by Hauschka et al. (3) in chicken bone and by Price et al. (4) in bovine bone. We have determined the complete covalent structure of the 49-residue bovine and human proteins that have three residues of the vitamin K-dependent amino acid, γ-carboxyglutamic acid; they are designated bone γ-carboxyglutamic acid-containing protein (BGP)[1] (5, 6). BGP accounts for 25% of the noncollagenous proteins extracted during demineralization of calf bone, an abundance which suggests that BGP is probably located in the extracellular bone matrix (4, 7). In vitro studies demonstrate that BGP binds strongly to hydroxyapatite, the mineral phase of bone, in an association that requires the γ-carboxyglutamic acid side chains (8, 9). We have previously shown that BGP is synthesized in calf cortical and cancellous bone culture and by rat osteogenic sarcoma cells in culture (7, 10). This newly synthesized protein is fully γ-carboxylated and is synthesized at a rate of about 1 BGP molecule/molecule of tropocollagen (7). Although

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[1] *Abbreviations used in this paper*: AP, alkaline phosphatase; BGP, γ-carboxyglutamic acid-containing protein of bone.

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the cells that synthesize the protein in bone have not been identified, the presence of 4-hydroxyproline at position 9 in the calf BGP sequence (5) shows that the protein has been modified by prolyl hydroxylase, an enzymatic marker used widely to distinguish osteoblasts from osteoclasts (11).

We have developed recently a radioimmunoassay for bovine BGP and have shown that this assay cross-reacts with purified human BGP (12). Studies with peptides of known structure derived from enzymatic digests of BGP indicate that this radioimmunoassay recognizes the COOH-terminal region of the 49-residue protein. With this assay, we have detected an immunoreactive component in bovine and human plasma identified as BGP by its identical radioimmunoassay dose-dilution curve and by its co-elution with BGP on Sephadex G-100 gel filtration (12). Experiments with addition of purified BGP to plasma show that plasma does not interfere with the quantitative measurement of added BGP. In addition, neither rat nor rabbit plasma displaces tracer from antibody, a result consistent with the specificity of this antibody for BGP from different species (12). Separate studies with another radioimmunoassay developed for rat BGP have also demonstrated the presence of BGP in rat plasma (13). As anticipated from the species specificity of this radioimmunoassay, the rat BGP tracer is not displaced from antibody by human, calf, or rabbit plasma. Thus, the results from analysis of three mammalian species with two independent radioimmunoassays consistently demonstrate the presence of high levels of BGP in plasma.

The present paper gives the results of our clinical studies on plasma BGP levels in normal subjects and in patients with bone disease (14).

METHODS

A total of 109 normal subjects and 120 patients with various bone disorders were studied after informed consent was obtained. With the exception of the patients with chronic renal disease, all had normal renal function. For all subjects and patients, a heparinized plasma sample was collected for biochemical determinations from an overnight fast. The normal adults had no evidence of calcium or skeletal abnormalities by routine history, physical, and biochemical evaluation. The patients with Paget's disease each had polyostotic involvement; a history, physical, biochemical evaluation; and x-ray and/or bone-scan findings diagnostic of this disorder. The patients with bone metastases all had biopsy-proven cancer (two adenocarcinomas of the lung, two small cell carcinomas of the lung, one adenocarcinoma of the breast) and metastases demonstrated by x ray. Primary hyperparathyroidism was established by surgery, and idiopathic hypoparathyroidism, by a consistent clinical presentation along with hypocalcemia, hyperphosphatemia, and undetectable parathyroid hormone (15). Liver disease was due to excess alcohol consumption and characterized by elevated serum aspartate aminotransferase, serum alanine aminotransferase, alkaline phosphatase, and bilirubin. Osteopenia was diagnosed by skeletal x rays including the presence of vertebral crush fractures and normal calcium and phosphorus.

BGP was measured by a radioimmunoassay procedure which has recently been described in detail and is thus now only outlined (12). Purified calf BGP was used for standard and tracer (4, 5). Tracer was prepared by lactoperoxidase radioiodination and purified by gel filtration on Sephadex G-25M. Rabbit antibody to BGP was used at a final dilution of 1:4,000. All assays contained (*a*) either a known amount of unlabeled BGP in 0.1 ml of assay diluent or varying amounts of heparinized plasma sample up to 200 μl; (*b*) 0.125 μl of antiserum and 2.5 μl of normal rabbit serum in 0.1 ml of assay diluent; (*c*) 15,000 cpm of ^{125}I-labeled BGP in 0.1 ml of assay diluent; and (*d*) sufficient assay diluent (0.14 M NaCl, 0.01 M phosphate, 0.025 M EDTA, 0.1% gelatin, 0.1% Tween 20, pH 7.4) for a final incubation volume of 500 μl. Assay mixtures were incubated in 10 × 75-mm borosilicate glass test tubes for 20 h at 25°C. The assay was terminated by the precipitation of rabbit antibody through the addition of 1.9 U of goat antiserum to rabbit γ-globulin (Calbiochem Behring Corp., American Hoechst Corp., San Diego, Calif., Lot 860217) in 0.1 ml of assay diluent. Subsequently the incubation was centrifuged to sediment ^{125}I-labeled BGP bound to rabbit antibody, and the supernate was discarded. Background ^{125}I label which adhered nonspecifically to the precipitate or to the glass reaction tube was measured by incubating ^{125}I-labeled BGP and normal rabbit serum without specific antiserum, then by the usual second antibody precipitation. Total and antibody-bound ^{125}I-labeled BGP were determined by counting in a Nuclear-Chicago gamma counter (Nuclear-Chicago Corp., Des Plaines, Ill.) for times sufficient to achieve a 2% counting accuracy. The fraction of ^{125}I-labeled BGP bound to antiserum, B, is defined as counts per minute in precipitate minus counts per minute in background divided by total counts per minute in assay; B_0 is the value of B when no unlabeled BGP is present. The B_0 values for each radioimmunoassay reported here are the average of nine independent determinations, and the B values for all standards and unknowns are the average of three independent measurements.

Previous studies have shown that the immunoreactivity of human plasma BGP is not affected by four freeze-thaw cycles or by lyophilization followed by reconstitution with either water or radioimmunoassay diluent; that the level of BGP in human plasma is unchanged after 24 h at 25°C and after 72 h at 4°C; and that there are no differences in BGP level between heparinized plasma and serum samples obtained at the same time (12). The intra-assay variation is typically <10%; interassay variation evaluated by repeated measurement of human samples has been <15% in 40 assays over a 1-yr period (12).

Alkaline phosphatase was measured spectrophotometrically with *p*-nitrophenylphosphate substrate according to the manufacturer's (Sigma Chemical Co., St. Louis, Mo.) directions (1). The normal range in our laboratory was <70 IU/liter. Calcium was determined by atomic absorption spectrophotometry. Gel filtration of plasma samples was performed as previously described; experiments with BGP purified from human bone demonstrate that calf and human BGP coelute in these procedures (12).

RESULTS

Fig. 1 demonstrates a typical standard curve for the BGP radioimmunoassay. Because previous studies have shown that purified bovine and human BGP dis-

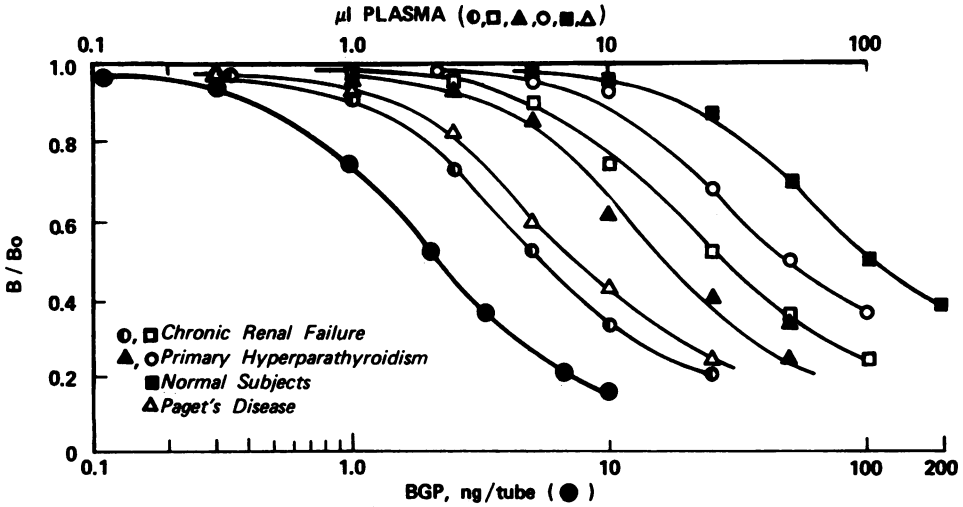

FIGURE 1 Radioimmunoassay of BGP. The reactivity in the assay of plasma samples was indistinguishable from that of the bovine BGP standard (●).

place bovine BGP tracer from antibody with equal effectiveness (12), the results obtained here using bovine BGP tracer and standard give the plasma concentration of human BGP directly. Several studies were undertaken to verify that the tracer displacement from antibody produced by plasma from humans is the result of the presence of BGP in plasma. As can be seen in Fig. 1, the reactions in the assay of the bovine BGP standard and that of plasma from patients with elevated levels of BGP are indistinguishable. Fig. 2 shows that the 50-fold elevated BGP level in plasma from an individual with Paget's disease of bone coelutes with pure BGP on Sephadex G-100 gel filtration. The small level of higher molecular weight immunogen is completely dissociated into BGP by incubation in 6 M guanidine HCl for 1 h at 25°C before a second filtra-

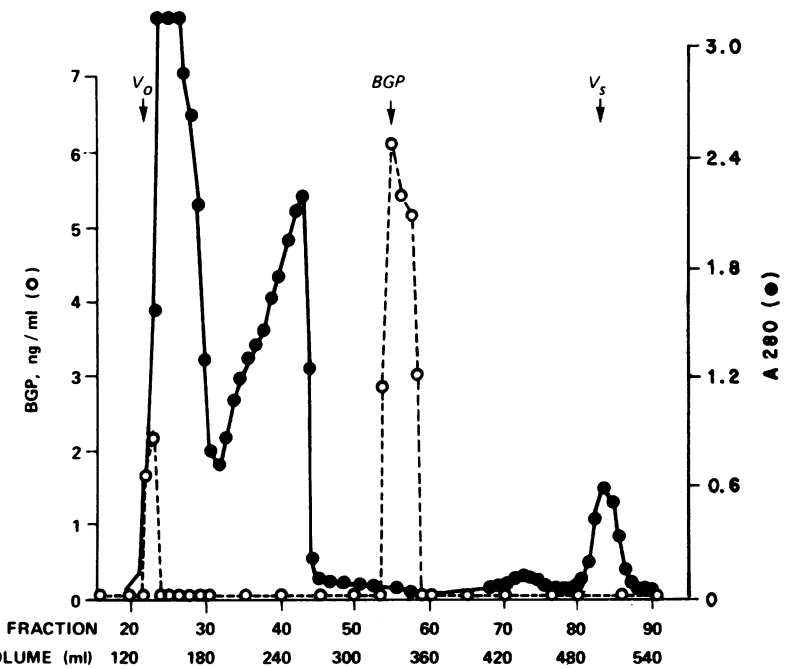

FIGURE 2 Gel filtration chromatography of 5 ml plasma from a patient with Paget's disease of bone on a 2 × 150-cm column of Sephadex G-100 eluted with 5 mN NH_4HCO_3 at 4°C. ○, BGP determined by radioimmunoassay on 0.1 ml of effluent. ●, A_{280}. Arrows indicate void volume (V_0), salt volume (V_s), and the elution position of purified calf BGP as determined in a subsequent chromatography on this column.

TABLE I
Relationships between BGP and AP in Human Blood

	BGP	AP	r	P
	ng/ml	*IU/liter*		
Normal adults (n = 109)	6.78(0.20)	26.7(1.3)	0.10	NS
Males (n = 47)	7.89(0.32)	28.1(2.1)	0.40	<0.005
Females (n = 62)	4.85(0.36)	25.2(1.8)	−0.17	NS
Paget's disease (n = 13)	39.2(16)*	587(181)*	0.66	<0.025
Bone metastases (n = 5)‡	15.8(2.6)*	132(26)*	0.93	<0.05
Hyperparathyroidism				
Primary (n = 11)	16.5(1.7)*	64(6.8)*	0.16	NS
Secondary (n = 38)§	47.3(6.1)*	56(8.5)*	0.64	<0.005
Hypoparathyroidism (n = 3)	2.40(0.92)*	—	—	—
Liver disease (n = 11)	5.77(1.0)	170(41)*	0.10	NS
Osteopenia (n = 20)	9.05(1.8)*	48(4.6)*	0.64	<0.01

Except as indicated and in osteopenic group, all subjects are male. Results are mean±SE.
* Significantly ($P < 0.05$–0.0005) different from normal controls.
‡ Squamous cell carcinoma of the lung (2), adenocarcinoma of the colon (2) and of the breast (2).
§ Chronic renal disease on hemodialysis.

tion. These results indicate that the elevated BGP level in plasma from patients with bone disease is, like BGP in normal bovine, human, and rat plasma (12, 13), probably identical to the 49-residue BGP found in bone.

Table I summarizes the comparison between BGP and alkaline phosphatase (AP) measurements in normal subjects and in patients with a variety of primary and secondary bone diseases. The patients with bone diseases characterized by increased bone resorption, and increased bone formation in some instances, had increased concentrations of both BGP and AP; the two were generally correlated. Both BGP and AP were higher in males than in females, but only BGP was significantly ($P < 0.01$) so. Table II summarizes the relationships among BGP, AP, and age in the normal subjects. For the females, there was a significant ($P < 0.005$) negative correlation ($r = -0.44$) between BGP and age, and a positive correlation ($r = 0.30$) between AP and age. BGP and AP were significantly ($P < 0.005$) correlated in males only. Both the BGP and AP in the male patients with Paget's disease were among the highest values detected and their levels were generally correlated.

The females with osteopenia (mean age 60 yr) had a mean concentration of BGP that was significantly greater than BGP in normal females (mean age 44 yr) (Table I). However, this group of patients also had concentrations of AP that were generally elevated. It is thus likely that the osteopenic group was heterogenous, and contained females with both osteomalacia and osteoporosis, because AP is not generally considered to be elevated in the latter (1).

The patients with bone metastases had increased levels of both BGP and AP, and the two were significantly ($P < 0.05$) correlated ($r = 0.93$). Positive correlations between calcium and BGP ($r = 0.54$) and AP ($r = 0.8$) did not achieve significance, perhaps because of the small number ($n = 5$) of patients. In the 11 males with primary hyperparathyroidism, BGP was clearly elevated above normal, whereas AP was within the upper limit of the normal range for this measurement. As indicated in Table I, however, the mean of AP for the patients was significantly greater than the mean for the normal males. Although three males with idiopathic hypoparathyroidism seemed to have decreased BGP, the number of patients was too small to be conclusive.

TABLE II
Significances and Correlations among BGP, AP, and Age in 109 Normal Subjects

	Males (n = 47)		Females (n = 62)		Both (n = 109)	
	r	P	r	P	r	P
BGP vs. AP	0.40	<0.005	−0.17	NS	0.10	NS
BGP vs. age	0.10	NS	−0.44	<0.005	−0.24	<0.005
AP vs. age	0.24	NS	0.30	<0.005	0.23	<0.01

TABLE III

Relationships among BGP, AP, Creatinine (Cr), and Blood Urea Nitrogen (BUN) in 38 males on Hemodialysis for Chronic Renal Failure

		r*		
	Mean±SE	AP	BUN	Cr
BGP, *ng/ml*	26.1(6.5)	0.64	0.54	0.56
AP, *IU/liter*	58.0(8.5)	—	0.39	0.49

Mean age of subjects was 64 yr.
* $P < 0.005–0.0005$.

Table III provides additional information about the 38 male patients with chronic renal disease. As with the patients with primary hyperparathyroidism, BGP was clearly elevated above normal, whereas the mean AP was within the normal range but significantly greater than in the normal males. BGP and AP correlated with each other and both correlated with blood urea nitrogen and creatinine. Table IV summarizes the BGP and AP measurements in 11 patients with rare bone disease.

DISCUSSION

BGP is a component protein of bone which is also present in plasma (4–8, 10, 12–14). Our studies demonstrate that the BGP measurement in plasma by radioimmunoassay may be a useful clinical procedure in evaluation of patients with bone disease. Several lines of evidence indicate that immunoreactive BGP in human plasma is indistinguishable from the bone BGP on which this immunoassay is based. Dose-dilution curves of human plasma samples in the assay react indistinguishably from the standard calf BGP (Fig. 1). The chromatographic elution position of human plasma BGP is also indistinguishable from that of the standard BGP (Fig. 2), as is that of calf serum (12). Calf plasma, but not rat or rabbit plasma, also displaces tracer from antibody, a result consistent with the immunochemical differences among these species of BGP (12). Conversely, a BGP assay based on the rat protein demonstrates immunoreactivity in rat plasma but not in human, calf, or rabbit plasma (13).

BGP is generally increased in the plasma of patients with diseases characterized by increased bone resorption and increased bone formation. Although BGP correlates with AP in some instances, there are many examples of dissociation between these two substances, which are produced by bone cells (Tables I–IV). BGP measurement has a theoretical advantage over AP measurement in this respect, since it seems to be a specific bone protein, whereas AP has several tissues of origin (1, 3, 5, 7). A dramatic example of this advantage is provided by patients with liver disease who have markedly elevated AP levels but essentially normal plasma BGP. Both AP and BGP seem to be related to age but in a different direction (Table II). Sex has a similar effect on both AP and BGP but the effect seems more dramatic on the latter (Tables I and II).

Despite these clinically promising observations, much further study is necessary to establish the value of BGP measurement in evaluation of the patient with bone disease. It will be necessary to correlate BGP measurements with additional biochemical tests such as hydroxyproline, with radionuclear procedures such as densitometry and quantitative radiography,

TABLE IV

BGP and AP Measurements in the Plasma of Patients with Uncommon Bone Diseases

		Patient No.				
Disorder		1	2	3	4	5
Engelmann's disease	BGP, *ng/ml*	65	17	30	19	13
	AP, *IU/liter*	132	55	12	17	19
Pseudohypoparathyroidism	BGP, *ng/ml*	16	16			
	AP, *IU/liter*	47	45			
Osteogenesis imperfecta	BGP, *ng/ml*	21				
	AP, *IU/liter*	181				
Fibrous dysplasia	BGP, *ng/ml*	63				
	AP, *IU/liter*	590				
Osteopetrosis	BGP, *ng/ml*	8.6				
	AP, *IU/liter*	48				
Mellorrheostosis	BGP, *ng/ml*	4.8				
	AP, *IU/liter*	39				

and with qualitative and quantitative histology. Serial measurements of BGP in patients with bone disease will be necessary to determine its clinical utility in assessing disease activity. Further studies will also be necessary to establish the relationship between BGP and bone-active substances such as parathyroid hormone, calcitonin, vitamin D metabolites, gonadal and adrenal steroids, and prostaglandins, to name a few. Such studies will assign to the measurement of BGP a proper place in the evaluation of bone function.

A better interpretation of plasma BGP measurement also depends on knowing the exact origin and metabolic fate of this peptide. It remains to be determined whether BGP comes from osteoblasts, osteoclasts, or some other cellular component of bone, although preliminary evidence suggests the first (5). Plasma BGP could thus arise from the release of newly synthesized BGP into plasma. Another source of plasma BGP could be bone extracellular matrix BGP, which is released upon bone resorption. BGP is a major bone matrix protein, and is readily released upon neutral or acid demineralization of bone (4, 7). The increase of plasma BGP in renal disease may be contributed to by decreased renal clearance of the peptide. Studies are in progress to identify both the origin of plasma BGP and its plasma clearance mechanism.

The presence of BGP in plasma and its elevation in bone diseases raises the possibility that BGP may play some role in the regulation of skeletal and calcium homeostasis. A role for BGP in bone mineralization and structure appears to be unlikely because of the absence of detectable abnormalities in bones from vitamin K-deficient animals, which have been <5% of the normal BGP level (9). An informational or hormonal function for BGP is supported by sequence features (5) such as the two pairs of basic residues, which are proteolytic cleavage sites in the activation of proteins such as proinsulin (16) and the pro-lys unit, which is present in many informational proteins (17). Studies are also in progress to test possible informational functions for the BGP (9, 18–20).

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