Increase in Serum Bone γ-Carboxyglutamic Acid Protein with Aging in Women

IMPLICATIONS FOR THE MECHANISM OF AGE-RELATED BONE LOSS

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ABSTRACT Because it is unclear whether age-related bone loss results from increased bone resorption, decreased bone formation or both, we measured the serum level of bone Gla-protein (BGP), a specific marker for bone turnover, in 174 women, ages 30 to 94 yr. Serum BGP increased linearly with aging ($r = 0.44$, $P < 0.001$) from 4.4±0.4 (mean±SE) in the 4th decade to 8.9±0.9 ng/ml in the 10th decade. This increase correlated inversely ($P < 0.001$) with concomitant decreases in bone mineral density at the lumbar spine, midradius, and distal radius. Using partial correlation coefficients, serum BGP still correlated positively with age ($r = 0.31$, $P < 0.001$) after creatinine clearance was fixed but not with creatinine clearance ($r = -0.04$, NS) when age was fixed. Urinary hydroxyproline ($r = 0.29$, $P < 0.001$), an index of bone resorption, and serum alkaline phosphatase ($r = 0.31$, $P < 0.001$), an index of bone formation, also increased with age and these increases correlated with increases in serum BGP ($r = 0.39$, $P < 0.001$ and $r = 0.43$, $P < 0.001$, respectively). Serum immunoreactive parathyroid hormone concentrations ($r = 0.39$, $P < 0.001$) and urinary cyclic AMP excretion ($r = 0.38$, $P < 0.001$) increased, suggesting that PTH secretion increased with age; these increases correlated significantly with increases in serum BGP. A subgroup of 32 women who were found to have vertebral fractures, hip fractures, or both had significantly higher values for serum BGP than the remainder. These data suggest that overall bone turnover increases in women with aging and, especially considering the concomitant decrease in skeletal mass, do not support the view that age-related bone loss results primarily from decreased bone formation.

INTRODUCTION

Bone mass decreases with aging in both women and men, but more substantially in women, a pattern that accounts for their greater susceptibility to osteoporosis. The characteristics of the abnormality of bone turnover that produces age-related bone loss are still controversial. Most studies attempting to define this abnormality have used quantitative analysis of bone biopsies. Using these methods, some investigators (1, 2) have found increased bone resorption with aging, whereas others (3–6) have found decreased bone formation. But these studies have important limitations. Most have used postmortem specimens, and thus had to use static rather than tetracycline-based, dynamic measurements of bone formation. Bone resorption rates, moreover, cannot be assessed by histomorphometry, and only static features such as resorption surfaces or number of osteoclasts can be quantified. Finally, in most studies, bone biopsy measurements have been restricted to trabecular bone, which represents only 20% of the skeletal mass (7).

Nevertheless, if the pathogenesis of age-related bone loss is to be understood, it is crucially important to resolve this controversy. As both cortical and trabecular bone are important for bone strength, it is mandatory to assess bone turnover of the entire skeleton. We have chosen, therefore, to study this problem by measuring a new marker for bone turnover, serum

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bone Gla-protein (BGP).[1] This compound, a 49-residue peptide that contains three residues of the vitamin K-dependent amino acid, γ-carboxyglutamic acid (Gla) (8–10), is unique to bone and constitutes 25% of the noncollagenous matrix proteins. Radioimmunoassay of plasma BGP (11) was found to be a sensitive test for bone turnover in patients with a variety of metabolic bone diseases (12). The demonstration that BGP is synthesized in vitro by rat osteogenic sarcoma cells (13) suggests that it is produced by osteoblasts. Moreover, a recent study by Price et al. (14) suggests that most or all of serum BGP is produced by cellular synthesis rather than by bone matrix degradation. 3 h after injection of rats with warfarin, an inhibitor of γ-carboxylation, BGP in serum was entirely decarboxylated, whereas BGP in bone was fully γ-carboxylated. Thus, intact circulating BGP seems to reflect mainly, if not entirely, bone formation.

We reasoned that if age-related bone loss is due to decreased bone formation it should be detected by this method. Measurements of serum BGP have been shown to have sufficient sensitivity to detect a decrease in bone turnover in hypoparathyroidism, although serum alkaline phosphatase was normal in these same patients (12). We also measured other, less specific markers for bone turnover serum alkaline phosphatase and urinary hydroxyproline.

METHODS

Subjects. From a randomized, stratified sample of the population of Rochester, MN, we selected 179 Caucasian women, 30–94 yr of age, as part of an ongoing epidemiologic study. All subjects gave informed consent. All were ambulatory, and none had a history of renal or hepatic disease. Five women were excluded because of the presence of a disease known to affect bone metabolism (hyperthyroidism, Paget's disease of bone, rheumatoid arthritis, and, in two, primary hyperparathyroidism). Of the remaining 174 subjects, 20 patients (subgroup A) had received thiazides (two patients), sex steroid hormones (three patients), thyroxine replacement for hypothyroidism or for simple goiter (13 patients), or intermittent low doses (<5 mg prednisone) of corticosteroids (two patients). All subjects 50 yr of age or older had roentgenograms of the spinal column and proximal femur. 32 of the randomly selected subjects (mean age 83 yr) were found to have fractures (subgroup B). Of these, 26 had only vertebral fractures, 15 had one or more collapsed vertebrae, defined as decreases of vertical height compared with adjacent vertebrae of >30%, and 11 had only wedging of one or more vertebrae, defined as decrease of 15 to 30%, and six subjects had a prior history of hip fracture (each of these had associated vertebral fractures).

Radioimmunoassay for serum BGP. We measured serum BGP using a radioimmunoassay system modified from that described by Price and Nishimoto (11). BGP was extracted and purified from bovine bone as previously described (9); its authenticity was assessed by amino acid composition, Gla-residue content, and the sequence of the first eight amino acids of the amino-terminal region of the molecule. This purified bovine BGP was used for standard and tracer. Both standard and tracer were homogeneous as assessed by SDS-gel electrophoresis. Tracer was prepared by chloramine-T radioiodination. Antiserum to bovine BGP was raised in rabbits as previously described (11) and used at a final dilution of 1:2,000. All assays contained (*a*) either a known amount of unlabeled BGP in 0.2 ml of assay diluent or varying amounts of serum samples up to 0.2 ml; (*b*) primary antiserum (R-102 M) added to 0.2 ml of a 1:40 dilution of normal rabbit serum in assay diluent; (*c*) 10,000 cpm of ^{125}I-labeled BGP in 0.1 ml of assay diluent (0.02 M Tris, 0.15 M NaCl, 2 mM Ca^{++}, 1% BSA, and 1% Triton, pH 8.0) for a final incubation volume of 0.5 ml. Assay mixtures were incubated in plastic test tubes (12 by 75 mm) for 3 h at 37°C. Rabbit antiserum were precipitated by adding two units of goat antiserum to rabbit γ-globulin (lot 160701, Calbiochem-Behring Corp., San Diego, CA) in 0.2 ml of assay diluent. After incubating for 14 h at 4°C, the tubes were centrifuged to sediment the ^{125}I-labeled BGP bound to the rabbit antibodies, and the supernatant was discarded. The pellet was washed with 1 ml of assay diluent and recentrifuged and the supernatant discarded. Nonspecific binding of ^{125}I-labeled BGP, measured by incubating ^{125}I-labeled BGP and normal rabbit serum without specific antiserum followed by the usual second antibody precipitation, is typically <1%. Total and antibody-bound ^{125}I-labeled BGP were determined by counting in a Beckman 6000 gamma counter (Beckman Instruments, Inc., Fullerton, CA) for 1 min. The fraction of ^{125}I-labeled BGP bound to antiserum ("B") was defined as counts per minute in precipitate divided by total counts per minute in assay; "Bo" was the value of B when no unlabeled BGP was present. The Bo values were the average of three independent determinations. B values for all standards were determined in triplicate, and B values for unknowns were the average of four independent measurements, namely, duplicate values at each of two dilutions of the sample (200 and 100 μl or 100 and 50 μl). The intra-assay variation is typically <10%; interassay variation evaluated by repeated measurements of human samples is <7%. The sensitivity of the assay is 0.2 ng of bovine BGP per tube (Fig. 1), and the

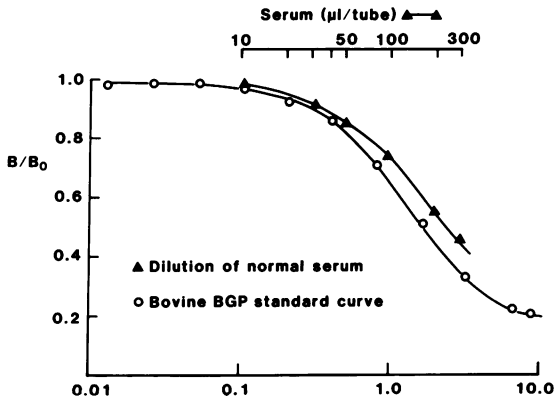

FIGURE 1 Radioimmunoassay of BGP showing similar reactivity of concentrations of bovine BGP standard (O—— O, nanograms per tube) and of dilutions of normal human serum.

[1] *Abbreviations used in this paper:* BGP, bone Gla-protein; BMD, bone mineral density; Gla, γ-carboxyglutamic acid; iPTH, immunoreactive parathyroid hormone.

concentration of BGP can be determined in >99% of normal subjects. Previous studies have shown that purified bovine BGP and human BGP displace BGP tracer from antibody with equal effectiveness (11). We verified that the presence of BGP in human plasma causes the displacement of tracer from our antibody by demonstrating that standard curves obtained with bovine BGP and with dilutions of human plasma were strictly parallel (Fig. 1). Gel filtration (Sephadex G-100) of serum from a patient with Paget's disease showed a single immunoreactive peak that coeluted with pure bovine BGP (Fig. 2).

Biochemical measurements. We obtained fasting morning blood samples for determinations of serum concentrations of calcium, creatinine, alkaline phosphatase, BGP, and immunoreactive parathyroid hormone (iPTH). Urinary hydroxyproline and creatinine clearance were determined in a 24-h collection, during which the subjects consumed a gelatin-free diet. cAMP was determined on a 2-h fasting morning urine specimen collected after oral hydration (400 ml). Serum calcium was measured by atomic absorption. Total alkaline phosphatase (15) was measured spectrophotometrically with *p*-nitrophenylphosphate substrate (Sigma Chemical Co., St. Louis, MO). Duplicate determinations of total alkaline phosphatase were performed for all samples, and the coefficient of variation was <10%. Creatinine concentrations in plasma and urine were measured by standard autoanalyzer (Technicon Instrument Corp., Tarrytown, NY) techniques. The glomerular filtration rate was assessed by 24-h creatinine clearance corrected to 1.73 m^2 of body surface area. Total urinary hydroxyproline was measured by the method of Kivirikko et al. (16) and was expressed as milligrams per deciliter of glomerular filtrate. Serum iPTH was measured by a modification of the method of Arnaud et al. (17). Crude extract from adenomatous parathyroid cells was used as a standard. A sheep anti-PTH antiserum (SH-1M) was used at a final concentration of 1:7,500. The intraassay variation was <15%, and the interassay variation was <20%. The concentration at which 50% inhibition of binding of ^{125}I-bovine PTH occurred was 50 pg for human PTH (1-84) and 1 ng for the 44-68 human fragment. The antiserum did not react with the 1-34 and 53-84 human fragments (18). Urinary cAMP was measured by a competitive protein-binding radioimmunoassay (kit purchased from Becton, Dickinson Immunodiagnostics, Orangeburg, NY). Values were expressed as millimoles per deciliter of glomerular filtrate. The coefficient of variation was <15%.

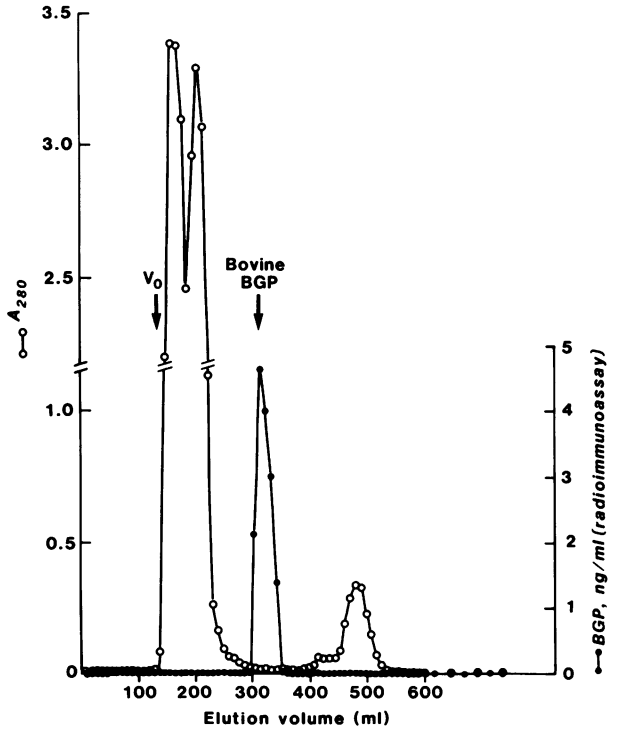

FIGURE 2 Chromatography of serum from a patient with Paget's disease of bone. 5 ml of serum were gel-filtered on a Sephadex G-100 column (2.6 × 90 cm) eluted with 50 mM NH_4HCO_3 at 4°C. Arrows depict void volume (V_0) and the elution position of purified bovine BGP. O, absorbance at 280 nm; ●, immunoreactive human BGP determined by radioimmunoassay on 0.1 ml of effluent. The elution position of purified bovine BGP was determined by a subsequent chromatography on the same column.

Bone densitometry. Bone mineral density (BMD) was determined at the midradius and distal radius, 2 cm proximal to the styloid process, by single photon absorptiometry (19). In our laboratory, this technique has a coefficient of variation of 3% for the midradius and 3–5% for the distal radius (20). BMD of the lumbar spine (L_1-L_4) was determined by dual photon absorptiometry; we used our modification (21, 22) of the method of Mazess et al. (23). For this method, the coefficient of variation is 2.3%.

Statistical analysis. Changes in variables with age were analyzed by linear regression analysis. Comparisons within different variables were investigated by multiple regression analysis that included partial correlation coefficients. Two-sample unpaired *t* tests were used to compare subgroups. All *t* tests were two-tailed.

RESULTS

Biochemical variables by decades are given in Table I and Figure 3. Correlations among these are given in

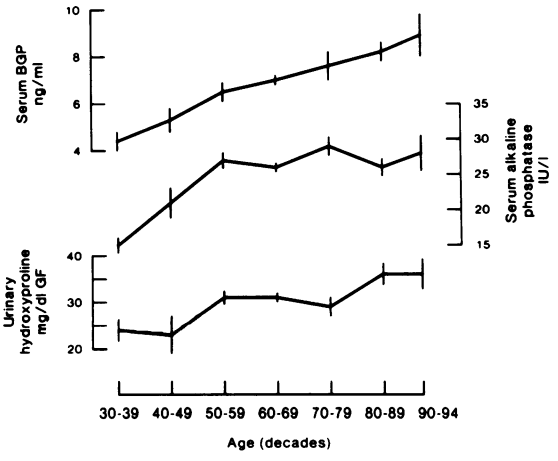

FIGURE 3 Mean values per decade for serum BGP, serum alkaline phosphatase and urinary hydroxyproline in 174 women.

TABLE I
Mean Values (±SE) per Decade for Serum BGP and Other Relevant Biochemical Measurements in 174 Women

Age range and mean age per decade	No. of subjects	Serum BGP	Serum alkaline phosphatase	Urinary hydroxyproline	Serum iPTH	Urinary cAMP	24-h creatinine clearance
yr		*ng/ml*	*IU/liter*	*mg/dl GF*	*μeq/ml*	*mmol/dl GF*	*ml/min*
30–39 (35)	16	4.4±0.4	15±1.0	24±2.3	44±4.9	2.68±0.10	99±5.0
40–49 (45)	12	5.3±0.5	21±2.1	23±3.9	81±10.8	2.71±0.21	93±7.4
50–59 (55)	45	6.5±0.4	27±1.1	31±1.4	60±3.6	3.45±0.16	90±3.1
60–69 (65)	35	7.0±0.2	26±0.6	31±0.9	80±3.3	3.48±0.08	82±2.7
70–79 (74)	19	7.6±0.6	29±1.3	29±2.0	96±10.5	3.87±0.19	68±3.5
80–89 (85)	39	8.2±0.4	26±1.2	36±2.3	99±6.9	3.99±0.17	52±2.9
90–94 (92)	8	8.9±0.9	28±2.5	36±3.2	101±12.5	3.69±0.16	37±7.3

Tables II and III. There was no significant regression of serum calcium with age. Serum BGP increased linearly with age; the predicted mean at age 90 yr was 95% more than the predicted mean at 30 yr. Serum alkaline phosphatase and urinary hydroxyproline also increased with age; for serum alkaline phosphatase, the predicted mean at age 90 yr was 30% more than the predicted mean at age 30 yr and for urinary hydroxyproline the predicted mean at age 90 yr was 56% more than at age 30 yr. In addition, significant increases with age were also found for serum iPTH and urinary cAMP. The 20 women taking medications (subgroup A) had mean values for BGP, alkaline phosphatase, iPTH, cAMP, urinary hydroxyproline, and BMD that were not significantly different from the other subjects. For the 32 women with vertebral or hip fractures (subgroup B), serum BGP concentration was higher (9.1±0.5 vs. 7.4±0.4, $P = 0.01$) and values for BMD at the lumbar spine were lower (0.83±0.03 vs. 0.97±0.03, $P < 0.001$) than 32 age-matched women without fractures. Other biochemical and densitometric findings were not different between patients with and without fractures.

TABLE II
Interrelationship of Age, Renal Function, and Serum BGP

	Correlation coefficient	
	Simple	Partial
Age vs. creatinine clearance	$r_{xy} = -0.67$*	
Age vs. serum BGP	$r_{xz} = -0.44$*	$r_{xz \cdot y} = -0.32$*
Serum BGP vs. creatinine clearance	$r_{zy} = -0.32$*	$r_{zy \cdot x} = -0.04$

x, age; y, creatinine clearance; z, serum BGP.
* $P < 0.001$.

Glomerular filtration rate, assessed by creatinine clearance, decreased significantly with age. The interrelationship of serum BGP, age, and creatinine clearance was analyzed by use of partial correlations. When creatinine clearance was held constant, serum BGP was correlated significantly with age. When age was held constant, serum BGP no longer significantly correlated with creatinine clearance (Table II). Bone density decreased with aging at all three scanning sites. Serum BGP, serum alkaline phosphatase, serum iPTH, and urine hydroxyproline correlated inversely with BMD at all scanning sites.

DISCUSSION

We found that serum BGP increased linearly with aging in a sample of women who were randomly selected from a community population and, thus, were representative of the general population of women. This increase occurred in spite of a concomitant decrease in bone mass. The decrease in BMD throughout life in women has been shown to be 47% for lumbar spine, 58% for femoral neck, 30% for midradius, and 39% for

TABLE III

Correlation Coefficients Between Age, Biochemical, and Densitometric Variables

	Age	BMD			Urinary cAMP	Serum iPTH	Urinary hydroxyproline	Serum alkaline phosphatase
		Lumbar spine	Mid-radius	Distal radius				
Serum BGP	0.44*	−0.45*	−0.40*	−0.46*	0.17‡	0.36*	0.39*	0.43*
Serum alkaline phosphatase	0.31*	−0.34*	−0.26*	−0.30*	0.16‡	0.27*	0.38*	
Urinary hydroxyproline	0.29*	−0.18‡	−0.23§	−0.25*	0.25*	0.26*		
Serum iPTH	0.39*	−0.20§	−0.32*	−0.32*	0.15‡			
Urinary cAMP	0.38*	−0.05	−0.14	−0.16‡				
BMD								
Lumbar spine	−0.47*							
Midradius	−0.67*							
Distal radius	−0.67*							

* $P < 0.001$.

‡ $P < 0.05$.

§ $P < 0.01$.

distal radius (22, 24). Although increased serum BGP with aging could reflect either increased production or decreased plasma clearance of BGP, we believe that the former possibility is more likely. First, we found that other markers for bone turnover, serum alkaline phosphatase, and urinary hydroxyproline, increased with aging too and were positively correlated with the increase in serum BGP. Second, all three markers correlated inversely with the age-related decrease in BMD. Third, although renal excretion appears to be the primary mechanism of clearance of BGP from the circulation (13), we could not demonstrate an inverse correlation with creatinine clearance when age was held constant. Also, in patients with various degrees of renal failure we[2] have found that serum BGP does not increase until glomerular filtration rates fall below 30 ml/min per 1.73 m^2. Thus, decreased plasma clearance resulting from decreased glomerular filtration rates with aging does not seem to be a major cause of the increased serum BGP.

In contrast to our findings, Price and Nishimoto (11) have reported a negative correlation of serum BGP with age in normal women but not in normal men. Because our radioimmunoassay system is very similar to theirs (10), we believe that this discrepancy may have resulted from their selection of subjects. Only 34 of the 62 normal women in their study were >30 yr of age and only 4 of them were >70 yr of age.[3] Radial bone growth continues for a decade after cessation of linear growth (25). For this reason, we did not include women in the decade 20–29 yr.

It is unclear how our findings can be reconciled with those from histologic studies of iliac crest biopsy samples that have shown age-related decreases in bone-forming surfaces (3, 4), in osteoblast counts (4) and in the mean wall thickness of trabecular bone packets (5). One possibility worthy of investigation, however, is that bone turnover in trabecular and cortical components of bone are affected differently by the aging process. Serum BGP measurements reflect the activity of the entire skeleton (which is composed of 80% cortical bone but only 20% trabecular bone [6]). By contrast, the histomorphometric studies that showed a decrease in bone formation were restricted to trabecular bone and did not assess bone turnover at the endosteal envelope, the process that accounts for the thinning of bone cortex with aging. In an analysis of >600 specimens of rib, Frost (26) found that the number, but not the thickness, of osteoid seams in cortical bone increased threefold between ages 30 and 70 yr; this finding is consistent with increased bone turnover and is not consistent with a decrease in bone formation. In contrast to the findings in cortical bone, Merz and Schenk (4) found that osteoid volume in trabecular bone of the iliac crest was unchanged with aging. Resorption of rib cortex also has been found to increase with aging (27). Microradiographic analysis of iliac crest biopsy samples, which included both cortical and trabecular bone, showed that bone resorption surfaces increased with aging (1). Furthermore, radiocalcium kinetic studies have shown that menopause increased bone turnover in the entire skeleton (28). Finally, bone densitometric studies (22, 29) have shown that the pattern of cortical and trabecular bone loss in women with aging was different.

The women with fractures were part of the random sample from the community population, and, thus,

[2] Delmas, P. D., D. M. Wilson, K. G. Mann, and B. L. Riggs. Unpublished observations.

[3] Price, P. A. Personal communication.

were free of referral bias. Because most of them were older than 80 yr, they should be classified as senile rather than postmenopausal osteoporosis. We recently showed that for patients with vertebral or hip fractures due to senile osteoporosis, mean BMD of the lumbar spine or proximal femur was only slightly less than that for age-matched normal controls (24). This is consistent with our finding that serum BGP in the elderly women with fractures was only slightly higher than in their normal peers.

In conclusion, the concordant results of three independent biochemical markers of bone metabolism—serum BGP, serum alkaline phosphatase, and urinary hydroxyproline—suggest that overall bone turnover increases in women with aging and may be increased even more in elderly women with fractures. Especially considering the concomitant decrease in skeletal mass, these data do not support the view that age-related bone loss results primarily from decreased bone formation.

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