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Serum Bone Gla Protein Variations During Estrogen and Calcium Prophylaxis of Postmenopausal Women

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Summary. We have evaluated serum bone Gla protein (BGP) changes in a double-blind study of early postmenopausal women during treatment with estrogen and calcium. To substantiate the changes in bone turnover, serum alkaline phosphatase, 24-hour urinary calcium, and bone mineral content (BMC) have also been measured. Our results indicate that serum BGP is a valuable measurement of bone metabolism.

Key words: Bone Gla protein — Postmenopausal bone loss — Estrogen.

Important tools in the management of age-related osteoporosis are reliable biochemical markers for bone turnover. Serum alkaline phosphatase has been used as an estimate of bone formation and urinary fasting hydroxyproline as an estimate of bone resorption. Neither measurement is entirely specific for bone turnover [1, 2]. Recently, a new marker for bone metabolism was identified [3]. The marker is bone Gla protein (BGP), the 49 residue protein of bone, which contains gamma-carboxyglutamic acid. It is the most abundant noncollagenous protein of bone and probably reflects osteoblast activity [4, 5]. BGP can now be determined in human blood by radioimmunoassay (RIA).

The aim of the present double-blind study was to measure BGP in early postmenopausal women before, during, and after treatment with either estrogen plus calcium or calcium alone. Moreover, other biochemical markers of bone formation and bone resorption, and bone mineral content (BMC) were determined simultaneously to compare the effects of the treatment on bone turnover and bone mass.

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Materials and Methods

Participants

The population was comprised of 80 postmenopausal women selected at random from a larger population studied to investigate the effect of estrogen on postmenopausal bone loss [6, 7]. In the former study, the participants were divided into four groups by random sampling and received different doses of sequential female hormones (17β-estradiol, estriol, and norethisterone acetate) and placebo. Moreover, all participants received 0.5 g calcium per day (Calcium Sandoz®). Two groups from the earlier study were pooled to form group 2 in the present study. These groups received estrogen in high and medium doses, and our previous investigation did not find significant differences in calcium metabolic parameters and BMC between the groups [7]. For treatment combinations, see Table 1. Of these 80 women, 39 (10 from group 1, 20 from group 2, and 9 from group 3) were selected by random sampling for serum BGP measurement. All participants had passed a natural menopause 6 months to 3 years earlier, and all were free of diseases or medication known to influence calcium metabolism. All were informed verbally and in writing about the trial and all gave their written consent. More details are given elsewhere [6, 7].

BGP Radioimmunoassay

The BGP radioimmunoassay (RIA) was performed as described elsewhere [8], except the samples are serum. hBGP37-49 and a monotyrosine analog (Tyr^{38}, $Phe^{42,46}$)hBGP38-49 were synthesized by Peninsula Laboratories (San Carlos, CA) using the solid-phase method of Merifield [9], purified by gel filtration chromatography, and verified by amino acid analysis, thin-layer chromatography, and high-voltage electrophoresis. Other re-

Table 1. Variation of bone metabolic parameters during the therapeutic trial

	No.	Initial values mean ± SD	After 24 months mean ± SD	After 36 months mean ± SD	After 42 months mean ± SD
Group 1					
Serum alkaline phosph. (U/liter)	20	146 ± 31	109 ± 29	113 ± 36	148 ± 31
24 h urinary Ca/Cr. (mmol/mmol)	20	0.631 ± 0.223	0.377 ± 0.151	0.479 ± 0.190	0.624 ± 0.203
BGP (pmol/ml)	10		9.1 ± 1.5	10.9 ± 1.9	14.1 ± 3.6
Treatment		Horm. + Ca	Horm. + Ca	Horm. + Ca	No treatment
Group 2					
Serum alkaline phosph. (U/liter)	40	141 ± 33	150 ± 35	107 ± 26	148 ± 48
24 h urinary Ca/Cr. (mmol/mmol)	40	0.640 ± 0.226	0.617 ± 0.189	0.481 ± 0.185	0.565 ± 0.192
BGP (pmol/ml)	20		12.5 ± 4.6	10.5 ± 3.9	13.9 ± 5.4
Treatment		Ca	Ca	Horm. + Ca	No treatment
Group 3					
Serum alkaline phosph. (U/liter)	20	156 ± 60	156 ± 63	150 ± 52	159 ± 54
24 h urinary Ca/Cr (mmol/mmol)	20	0.602 ± 0.232	0.573 ± 0.232	0.569 ± 0.189	0.584 ± 0.163
BGP (pmol/ml)	9		11.2 ± 2.5	12.1 ± 3.0	15.0 ± 2.5
Treatment		Ca	Ca	Ca	No treatment

agents and their sources were keyhole limpet hemocyanin (Calbiochem, San Diego, CA); ethyl, dimethylaminopropyl carbodiimide, beta D-glucose, glucose oxidase (Sigma, St. Louis, MO); $Na^{125}I$ (Amersham, Arlington Heights, IL); lactoperoxidase (PL Biochemicals, Milwaukee, WI); rabbit gamma globulin (Miles Biochemicals, Elkhart, IN); and goat antirabbit gamma globulin (Antibodies, Inc., Davis, CA).

Immunization

hBGP37-49 (1.5 mg) was conjugated to 1.8 mg keyhole limpet hemocyanin in phosphate-buffered saline using two additions of 250 μg of ethyl, dimethylaminopropyl carbodiimide at room temperature. After 24 h, the material was dialyzed against water and frozen in aliquots at −20°C. Four rabbits were injected with 50 μg of conjugate in complete Freund's adjuvant and boosted with an equal amount of incomplete Freund's adjuvant at monthly intervals. In 2 rabbits, high-affinity antisera developed and gave maximal titers at 3 months.

Radioligand

1.0 μg of (Tyr^{38}, $Phe^{42,46}$)hBGP38-49 was iodinated in 0.05 M phosphate buffer with 1 mCi of $Na^{125}I$ by 2 μg lactoperoxidase in the presence of 2 μmol of beta-D-glucose and 80 μU of glucose oxidase (125 U/mg). After 15 min at room temperature, the reaction mix was stopped by dilution with 1 ml 0.1 M NaH_2PO_4 pH 2.7 containing 100 μg cytochrome C carrier protein. The radioligand was purified by adsorption to a octadecasilyl silica cartridge (C18 SepPak, Waters Assoc., Milford, Mass.). The cartridge was washed with 10 ml 0.1 M NaH_2PO_4 pH 2.7, 10 ml 0.1% trifluoroacetic acid (TFA) and 10 ml 0.1% TFA in methanol/H_2O (40:60). The radioligand was eluted with 0.1% TFA in 80% methanol/H_2O and stored at −70°C.

BGP-depleted Plasma

Blood bank plasma was treated with 16 U/ml of heparin, dialyzed thrice against 10 volumes of 10 mM $CaCl_2$, thrice against 10 volumes of water, lyophilized, and reconstituted at 8 g/dl in normal saline. This preparation is free of BGP as assessed by gel filtration chromatography on BioGel P30 followed by RIA.

Assay

Ten microliters of heparin plasma sample, or hBGP37-49 standards (1–30,000 femtomol) with 10 μl of BGP-depleted plasma, was mixed with rabbit anti-BGP antibody (1:10,000), radioligand (4–6 nCi) and 20 μg rabbit gamma globulin in 0.25% BSA, 1 mM EDTA, 20 mM sodium phosphate buffer, pH 7.4, and incubated at 4°C for 3 days (500 μl final volume). Goat antirabbit gammaglobulin (7 μl) was then added in 0.7 ml assay buffer and 16 h later the tubes were centrifuged, decanted, and counted. Results were expressed as picomoles of immunoreactive hBGP37-49 per ml.

Human BGP Reactivity

Human BGP was purified to a single peak by HPLC, with monitoring by absorbance at 214 nm and RIA. Except during chromatography, BGP was kept at −20°C. Five grams of human rib bone were obtained at autopsy and kept at −70°C. The bone was cleaned of soft tissue, ground in 20% formic acid, and stirred at 4° overnight. The extract was adjusted to 1% TFA, allowed to stand at 4°C for 1 h, and centrifuged at 10,000 × g × 1 h. The supernatant was applied to octadecasilyl silica cartridge (C18 SepPak), washed with 0.1% TFA, and eluted with 5 ml 0.1% TFA in 80% methanol. The eluates were dried with a nitrogen stream, dissolved in 0.1 M NaH_2PO_4 pH 2.7, injected on an

Aquapore RP300 column (0.4 × 30 cm) equilibrated with the same solvent and eluted with an isopropanol gradient (0–60%, 1% per min). The BGP peak was reinjected on a Vydac 218TP column in 0.1% TFA, and eluted with an acetonitrile gradient (6–60%, 1% per min), and finally rechromatographed on a shallow acetonitrite gradient (30–36% over 45 min). The identity and concentration of the BGP were determined by amino acid analysis; 28 nmol were recovered. From this material, the three tryptic fragments were also prepared and rechromatographed on the Vydac HPLC column. In the absence of plasma, the potency of hBGP in the radioimmunoassay was 0.56 times that of hBGP37-49 on a molar basis, very similar to the figure of 0.52 times previously obtained for intact bovine BGP [8]. Tryptic fragments were not reactive. To estimate the mean normal plasma BGP level in ng hBGP/ml, a master standard of hBGP37-49 was prepared (sufficiently concentrated for amino acid analysis) and the plasma BGP assay was calibrated against dilutions of this and hBGP. The mean normal plasma BGP was estimated to be 13.5 ng/ml. However, we have observed that serum samples give higher measurements of BGP than matched plasma samples, as is the case in this study, and we are in the process of evaluating these differences [8].

Bone Mineral Content

BMC was measured by photon absorptiometry (^{125}I) and was calculated as the mean of 12 scans, six on the distal part of each forearm. BMC is expressed in arbitrary units (dimension: g/cm). This method has a long-term reproducibility of 1.2% in normal subjects, and values correlated well with BMC at other sites and with total body calcium [10].

The concentrations of serum *calcium* and *alkaline phosphatase*, and *fasting urinary calcium* and *creatinine* were measured by standard procedures.

After 12 h overnight fast, the participants emptied their bladder between 8 and 9 AM. Sixty minutes later, a urine sample was obtained for determination of calcium and creatinine. Calcium excretions relative to creatinine were obtained from the concentration ratio and expressed in mmol/mmol.

The intra-individual biological variation of calcium metabolic indices was calculated as the intra-individual changes in group 3 (the calcium group) between 24 and 36 months. These were 11.8% for serum BGP, 8.5% for serum alkaline phosphatase, and 22.6% for urinary calcium/creatinine.

Statistical analysis was done with a paired Student's *t* test.

Results

The concentrations of BGP are given in Table 1 and Fig. 1. The mean values of groups 2 and 3 at 24 months (without hormone treatment) were significantly higher than the corresponding mean values in group 1 on estrogens ($P < 0.01$). During continued hormone treatment, the serum BGP was almost unchanged. In group 2, which was initially treated with calcium alone and thereafter with hormones plus calcium, the mean serum BGP fell significantly after 12 months when hormones were administered ($P < 0.01$), and only 4 values did not fall. In all three groups the mean BGP increased signficantly after withdrawal of treatment ($P < 0.01$ and $P < 0.001$). The other biochemical markers for bone turnover are given as mean ± 1 SD in the table. The changes in serum alkaline phosphatase and 24 h urinary calcium tend to coincide with the changes in BGP except in group 3 when the calcium intake was withdrawn.

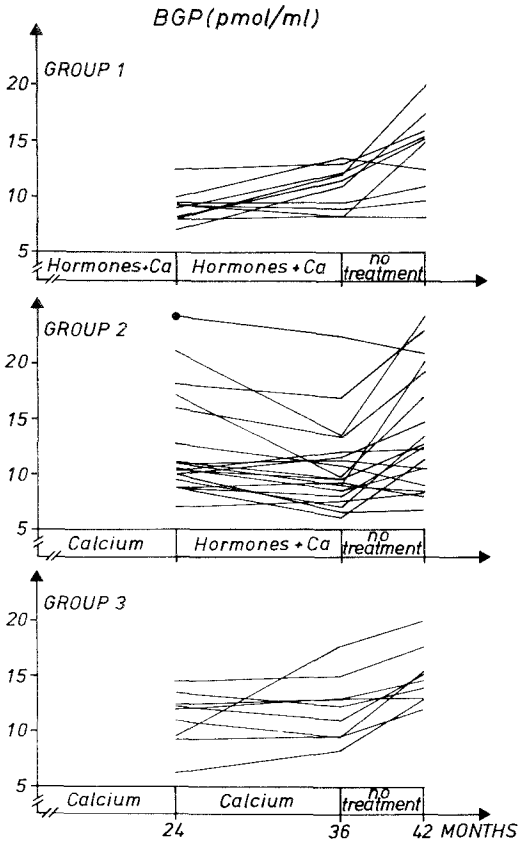

Fig. 1. Individual changes of serum BGP in each of the three groups during the therapeutic trial.

Figure 2 shows the changes in bone mass. The fall in BMC in group 3 after withdrawal of calcium is parallel to the decrease in the other two groups after withdrawal of hormones plus calcium. Further, there is a highly significant increase during treatment with hormones (groups 1 and 2, 24–36 months, $P < 0.01$), a significant decrease during treatment with calcium (group 3, 24–36 months, $P < 0.05$), and a decrease after withdrawal of hormones at the same magnitude as in the calcium group (or no treatment group) [11].

Discussion

The study has demonstrated that BGP is a useful

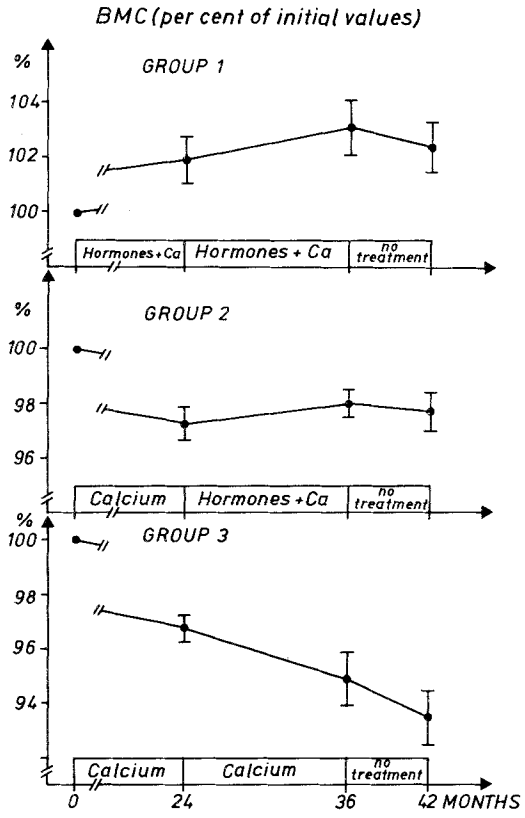

Fig. 2. Changes in BMC in each of the three groups (mean ± 1 SD) during the therapeutic trial. Note that the abscissa is broken between time t_0 and t_{24}. The increase in group 1 in BMC for the first 2 years was 2%, and the decrease in groups 2 and 3 was in the order of 3%.

biochemical marker of bone metabolism. Previous investigations have indicated that BGP most probably reflects bone formation or osteoblast activity [4, 5]. In some studies, serum alkaline phosphatase is used to monitor this parameter. However, serum alkaline phosphatase is not specific for bone formation because its concentration in blood also receives contributions from other sources. On the other hand, if a valid determination of isoenzyme is available, the specificity will probably be increased. Serum BGP in normal subjects and in various smaller groups of patients with various diseases known to influence bone metabolism has been determined in previous studies [3]. Further, serum BGP variations have been determined during short-term treatment of the same patients [13].

In this study we have evaluated the BGP variations in a double-blind controlled therapeutic trial primarily performed to evaluate the effect of estrogen on postmenopausal bone loss [6, 7]. This effect is due to a reduction of both bone resorption and bone formation. The decrease in bone formation has clearly been demonstrated and it does not exceed the premenopausal level. The increase in BMC during estrogen treatment must be due to a greater reduction in bone resorption than in bone formation.

Serum BGP increased highly significantly on discontinuation of both hormones plus calcium and calcium alone. The effect of calcium on bone metabolism could not be shown when applying serum alkaline phosphatase or BMC measurements. This suggests that serum BGP is a sensitive parameter for changes in bone turnover.

The levels of immunoreactive BGP in normal human plasma measured by our assay are somewhat higher than those reported for native assays using antisera to bovine BGP [3, 12, 13]. Studies of gel filtration of plasma in this and other laboratories [8, 12] indicate that some plasma BGP may be weakly bound to other proteins in blood. Such binding may explain the higher concentrations of BGP detected by our assay since the smaller sample volumes would facilitate dissociation of these complexes and binding of BGP to specific antibodies. Interference by plasma proteins has also been observed in an RIA using antibody to native bovine BGP [14]. We control for this plasma protein effect with a solution of BGP-depleted plasma proteins. In other BGP assays, serum samples are assayed in the presence of detergents [12]. We believe levels of BGP in blood assessed by RIAs including ours, can be considered only estimates of the true concentration.

The present data indicate that determination of serum BGP may be a cheap and valuable estimate of successful prophylactic treatment of postmenopausal osteoporosis, but further studies are of course necessary, and are in progress in our department.

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References

1. Poser S, Cornish C, Kleerekoper M (1977) Alkaline phosphatase and metabolic bone disorders. In: Aviol LV, Krane SM, (eds) Metabolic bone disease. Academic Press, New York, pp 141–181
2. Prockop DJ, Kivirikko KI, Tuderman L, Guzman NA (1979) The biosynthesis of collagen and its disorders. N Engl J Med 301:13–23
3. Price PA, Parthemore JG, Deftos LJ (1980) New biochemical marker for bone metabolism. J Clin Invest 66:878–883
4. Price PA, Poser JW, Raman N (1976) Primary structure of

the gamma-carboxyglutamic acid-containing protein from bovine bone. Proc Natl Acad Sci 73:3374–3375
5. Price PA, Williamson MD, Lothringer JW (1981) Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. J Biol Chem 256:12760–12766
6. Christiansen C, Christensen MS, McNair P, Hagen C, Stocklund K-E, Transbøl I (1980) Prevention of early postmenopausal bone loss: controlled 2-year study 315 normal females. Eur J Clin Invest 10:273–279
7. Christiansen C, Christensen MS, Larsen N-E, Transbøl I (1982) Pathophysiological mechanisms of estrogen effect on bone metabolism. Dose-response relationship in early postmenopausal women. J Endocrinol Metab 55:1124–1130
8. Catherwood BD, Marcus R, Madvig P, Cheung AD (in press) Determinants of bone and gamma-carboxyglutamic-acid containing protein in plasma of healthy aging subjects. Metabolic Bone Disease and Related Research
9. Merrifield RB (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. J Am Chem Soc 85:2149–2154
9a. Catherwood BD, (in press) Development and replication of radioimmunoassays for BGP. In: Cohn DV, Talmage RV, Mathews JL (eds) Proceedings of the 8th International Conference on Calcium Regulating Hormones, Excerpta Medica
10. Christiansen C, Rødbro P (1975) Estimation of total body calcium from the bone mineral content of the forarm. Scand J Clin Lab Invest 35:425–431
11. Christiansen C, Christensen MS, Transbøl I (1981) Bone mass in postmenopausal women after withdrawal of oestrogen/gestagen replacement therapy. Lancet i:459–461
12. Price PA, Nishimoto SK. Radioimmunoassays for the vitamin K-dependent protein of bone and its discovery in plasma. Proc Natl Acad Sci 77:2234–2238
13. Deftos LJ, Parthemore JG, Price PA (1982) Changes in plasma bone Gla protein during treatment of bone disease. Calcif Tissue Int 34:121–124
14. Gundberg CM, Cole DEC, Lian JB, Reade TM, Gallop PM (1983) Serum osteocalcin in the treatment of inherited rickets with 1,25-dihydroxyvitamin D_3. J Clin Endocrinol 56:1063–1067