Homologous Radioimmunoassay of Human Osteocalcin

Roger Bouillon,1 Dirk Vanderschueren, Erik Van Herck, Henning K. Nielsen,2 Marie Bex, Walter Heyns, and Hugo Van Baelen

Osteocalcin or bone γ-glutamic acid-containing protein (GLA protein) was isolated from human bone and used to develop a homologous radioimmunoassay of human osteocalcin. The effect of age on serum osteocalcin was studied in 380 normal children and adolescents and 330 normal adults. The mean (± SD) values in adults were higher in men [25 ± 5 μg/L (4.3 ± 0.8 nmol/L)] than in premenopausal women [20 ± 6 μg/L (3.4 ± 1.0 nmol/L); P < .01], but both were lower than in postmenopausal women [28 ± 2 μg/L (5.0 nmol/L)]. The highest concentrations were seen in girls [ages 10–12 years: 99 ± 38 μg/L (17.0 nmol/L)] and boys [ages 14–16 years: 107 ± 57 μg/L (18.4 nmol/L)]. These mean values were substantially higher than those previously reported for results of heterologous osteocalcin radioimmunoassays but the correlation (r = 0.87, n = 77, P < .001) between both sets of results was excellent. In patients with metabolic bone diseases characterized by high or low bone turnover, the increase or decrease in serum osteocalcin observed was as expected. This homologous radioimmunoassay of human osteocalcin thus reflects bone turnover but reports serum concentrations higher than previously suspected.

Additional Keyphrases: osteoporosis · bone GLA protein · vitamin D · bone metabolism · calcium metabolism · sex- and age-related effects · menopausal status

Osteocalcin (OC) or bone γ-glutamic acid-containing protein (GLA-protein) is a 5.8-kDa calcium-binding protein exclusively produced by osteoblasts and dentinoblasts. Its synthesis and secretion depend on both vitamin K and vitamin D (1–3). Although OC represents ~2% of total bone proteins, its precise function is not well understood. Serum concentrations of OC, however, correspond generally well with the activity of the cells producing it and thus with bone formation (1–10). Immunoassays of OC are therefore widely used to evaluate bone formation; OC is a marker of a more mature osteoblast function than is alkaline phosphatase (EC 3.1.3.1), although their concentrations in serum correlate well (11). RIAs of OC usually require species-specific antibodies (e.g., for rat, mouse, chick, and guinea pig), but human osteocalcin (hOC) was until recently usually measured by heterologous assays, in which ovine or bovine OC was used as label and standard, with anti-ovine or anti-ovine OC polyclonal (5, 9, 10, 12–18) or monoclonal (17–19) antisera. Because ovine and bovine OC differ from hOC by only five amino acids, antisera do not markedly differentiate between these forms of OC and therefore can be used in heterologous assays. RIAs based on anti-hOC antisera are rare, probably because of the greater difficulty in isolating OC from human bone (20–22). However, serum concentrations of OC reported with a homologous human assay (21) were markedly higher than when measured with heterologous assays (4, 23). We therefore purified OC from normal human bone, and developed and evaluated a homologous RIA performed with a polyclonal guinea pig antiserum.

Materials and Methods

Purification of human osteocalcin. hOC was purified according to a modification of the method of Gundberg et al. (24). Briefly, fragments of tibial head obtained from knee replacement surgery were cleaned of soft tissue, coarsely ground in liquid nitrogen until the particle size was <100 mesh, and extracted with 200 mL/L formic acid reagent (5 mL/g dry bone) at 4°C for 24 h. After centrifugation, the supernate was desalted on Sephadex G25 (Pharmacia, Uppsala, Sweden) equilibrated with the formic acid reagent, and lyophilized. We redissolved the lyophilisate in 50 mmol/L Tris · HCl buffer, pH 7.4, containing guanidine · HCl, 4 mol/L, and applied it to a Sephadex G50 column (Pharmacia) equilibrated with the same buffer. Fractions containing OC (determined by an in-house RIA of bovine OC (16)) were pooled, passed through a Sephadex G25 column equilibrated with Tris · HCl buffer (0.1 mol/L, pH 8), applied to a diethylaminoethyl (DEAE) cellulose column (DE 25; Whatman, Maidstone, Kent, UK), and finally eluted with a linear NaCl gradient (from 0.1 to 1 mol/L). The peak fractions containing OC were again pooled, passed through a Sephadex G25 column equilibrated with the same Tris buffer, and applied to an FPLC Mono Q cation-exchange column (Pharmacia), from which it was eluted with a NaCl gradient from 0.1 to 5 mol/L. The mass of the protein was obtained by amino acid analysis, and the absorptivity of the purified product was measured at 280 nm.

Antiserum. A guinea pig was immunized at monthly intervals by subcutaneous injection of 100 μg of hOC, previously coupled to thyroglobulin by the glutaraldehyde reaction and emulsified in complete Freund's adjuvant (Difco Labs, Detroit, MI) for the initial injection, or

Received March 16, 1992; accepted May 20, 1992.
of 35 μg in incomplete Freund’s adjuvant (four subsequent booster injections).

Iodination of human osteocalcin. hOC (5 μg) was iodinated by the Chloramine T method; the product had a specific activity of ~5000 GBq/g. The protein was further purified by gel chromatography on columns of PD10 and Sephadex G50, equilibrated with assay buffer (10 mmol/L sodium phosphate buffer, pH 7.4, containing 0.12 mol of NaCl, 25 mmol of sodium EDTA, 1 g of bovine serum albumin, and 1 mL of Tween 20 per liter).

Immunoassay. Incubate 50 μL of serum or standard with 600 μL of guinea pig anti-hOC antiserum (diluted 50 000-fold in assay buffer), sufficient to bind 40% of tracer. Add 100 μL of 125I-labeled hOC (~15 000 counts/min) and incubate overnight at 4 °C. Finally, separate the phases by adding 100 μL of donkey anti-guinea pig γ globulins coupled to CNBr-activated cellulose (SacCel; IDS, Washington, UK).

Subjects. Serum samples were collected from 207 healthy blood donors (82 men, ages 20–65 years, and 125 premenopausal women), from 34 healthy men older than 70 years, and from 89 postmenopausal women. The serum samples of postmenopausal women were kindly supplied by E.C.H. Van Beresteijn (NIZO, Ede, The Netherlands). Serum samples were also obtained from 380 healthy children and adolescents (ages 5–19 years) during a routine medical school examination after receiving written informed consent from the parents. Samples from patients with various diseases known to affect bone turnover were collected in the morning at the University Hospitals of Leuven, Belgium. Serum samples from 11 pregnant Danish women were collected at monthly intervals during and after pregnancy for 2 months postpartum. Serum samples from healthy blood donors and chronic renal failure patients were analyzed not only with the hOC assay but also with the in-house assay for bovine OC (16). Total alkaline phosphatase activity was also determined in these serum samples by AutoAnalyzer (Technicon, Tarrytown, NY) techniques.

Osteocalcin fragments. Fragments of hOC spanning amino acid sequences 1–10, 7–19, 25–37, and 37–49, and coded lyophilized serum samples used in a previous collaborative study on OC measurements (4), were kindly provided by P.D. Delmas (Lyons, France).

Statistical analysis. Student’s t-test and linear-regression analysis were used to compare OC values between different groups. All values are presented as mean ± SD.

Results
Purification and Characterization of hOC

A three-step purification procedure (Figure 1) yielded a pure protein, as demonstrated by ion-exchange chromatography and polyacrylamide gel electrophoresis with retention of γ-carboxyglutamic acids. The amino acid composition of the protein eluting as a single homogeneous peak corresponded with that of hOC. In accordance with earlier measurements, the estimated value for the absorptivity was ε₁₀ μmol/L = 13.3 (18). After dot-blotting analysis, we demonstrated the presence of γ-carboxyglutamic acid by a specific colorimetric method (25).

The yield (1 mg of protein from 100 g of bone) was lower than that for rat (26), chick (27), guinea pig, or mouse OC (unpublished personal results) because of the much smaller OC content in human bone.

Technical Characteristics of the hOC Assay

The homologous hOC assay allowed detection of 2 to 100 μg/L in a 0.05-mL serum sample, with a detection limit of 2 μg/L (defined from interpolation of the standard curve 2 SD below the B₀) (Figure 2). The within- and between-assay CVs were 4.5% and 8.6% for a low value (7.6 μg/L, n = 14) and 6.4% and 7.1% for a high value (36 μg/L), respectively. The validity of the assay was further confirmed by dilution and recovery experiments (Table 1, Figure 3). Ovine, bovine, horse, and goat OC were recognized by the hOC assay system, but
Fig. 2. Radioimmunoassay of human osteocalcin
Displacement of \(^{125}\text{I}\)-labeled hOC by purified hOC (\(\Theta\)) or bovine OC (+) and by various animal sera: ovine (+), bovine (*), goat (\(\odot\)), horse (\(\circ\)), chick (\(\ast\)), mouse (\(\Delta\)), rat (\(\square\)), and dog (\(\Box\)).

Table 1. Analytical Recovery Experiments in the Assay of hOC

<table>
<thead>
<tr>
<th>hOC added, ng</th>
<th>Analytical recovery, % of expected value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2.5</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>5</td>
<td>103 ± 3</td>
</tr>
<tr>
<td>15</td>
<td>102 ± 2</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SD (n = 7); original hOC content: 7.4 ± 1.3 µg/L (1.27 nmol/L).

Sera from these species did not dilute in perfect parallel fashion to hOC (Figure 2). The antiserum did not cross-react with chick, dog, mouse, or rat serum. Several synthetic hOC fragments also did not react with the OC assay system (Figure 3).

The presence or absence of calcium clearly influenced the apparent hOC concentration: the addition of CaCl\(_2\) or Na\(_2\)EDTA decreased the binding of \(^{125}\text{I}\)-labeled hOC (Figure 4). This effect of calcium was further evaluated by supplementing a normal serum sample (hOC content 20 µg/L) with increasing amounts of CaCl\(_2\) (from 0.2 to 12.8 g/L) before assaying the sample under the conditions described above. The apparent OC value was affected by the presence of added CaCl\(_2\) at 12.8 g/L (320 mmol/L), increasing to an apparent value of 25 µg/L, but was not influenced by lower calcium concentrations.

Osteocalcin Concentration in Human Serum

In healthy adult blood donors, serum OC concentrations fluctuated little between ages 25 and 50 years, with no significant sex difference (Figure 5). The mean (± SD) serum OC was significantly higher in men [25 ± 5 µg/L (4.3 ± 0.8 nmol/L), n = 64] than in women [20 ± 6 µg/L (3.4 ± 1.0 nmol/L); ages 38 ± 1 years, n = 80]. When several samples (n = 77) chosen without conscious bias were measured with a heterologous bovine OC assay (16), the corresponding values were 6.9 ± 2.4 µg/L (men) and 4.4 ± 1.8 µg/L (women); the correlation between both sets of results was excellent (Figure 6). In postmenopausal women the OC concentration was significantly greater [29 ± 2 µg/L (5.0 nmol/L); ages 64 ± 2 years; n = 89; P < 0.001] than in premenopausal women. In healthy elderly men (ages 78 ± 4 years), serum OC [23 ± 5 µg/L (3.9 nmol/L), n = 34] was not significantly different from that in younger adults. In normal children and adolescents serum OC was much higher, with peak values of 99 ± 38 µg/L (17.0 nmol/L) in girls (ages 10–12 years) and 107 ± 57 µg/L (18.4 nmol/L) in boys (ages 14–16 years) (Figure 5).

CLINICAL CHEMISTRY, Vol. 38, No. 10, 1992
In predialysis chronic renal failure, serum OC was markedly increased [69 ± 63 μg/L (11.9 nmol/L), n = 96, P < 0.001 vs normal adults]. A significant correlation was also observed with OC measured by a heterologous assay (r = 0.76; P < 0.001), with serum alkaline phosphatase (r = 0.32, P < 0.001), and with serum creatinine (r = 0.5, P < 0.001).

Decreased OC concentrations were observed in 11 normal pregnant women monitored prospectively in early pregnancy [at 3 months' gestation: 14 ± 0.6 μg/L (2.4 nmol/L)], but values were normal at delivery [20 ± 0.2 μg/L (3.4 nmol/L)] and 2 months later (Figure 7).

In situations with increased bone turnover, serum concentrations of OC were increased: Paget disease [36 ± 9 μg/L (6.2 nmol/L); n = 8], hyperparathyroidism [54 ± 33 μg/L (9.3 nmol/L); n = 24], and hyperthyroidism [44 ± 16 μg/L (7.6 nmol/L); 19 women and 8 men, ages 46 ± 4 years]. In comparison with results for age- and sex-matched control subjects, concentrations of serum OC were decreased in patients with biochemical vitamin D deficiency [18 ± 8 μg/L (3.1 nmol/L); n = 65] and during glucocorticoid treatment [17 ± 5 μg/L (2.9 nmol/L); n = 9]. Patients with postmenopausal osteoporosis [25 ± 7 μg/L (4.3 nmol/L), ages 67 ± 2 years, n = 41] had OC values similar to those of osteoarthritis patients [27 ± 6 μg/L (4.6 nmol/L), ages 67 ± 2 years, n = 18] or of normal control subjects of similar age.

When OC was measured in coded serum samples previously used in an interlaboratory comparative study of RIAs of OC, the present assay yielded higher concentrations. Although the relative agreement with other assays was good, the absolute concentration measured with the present hOC RIA agreed better with results by another homologous RIA than with the heterologous assays (Figure 8).

Discussion

We isolated osteocalcin from human bone samples in a three-step procedure, but the yield was lower than for...
similar extractions from animal bones. Others obtained similar results, which suggests a lower OC content in human bones than in animal bones (21, 28, 29). The gamma-carboxylated glutamic acids were retained in the purified protein, and the absorptivity of the protein agreed well with a previous estimation (18).

The RIA of hOC followed a classical procedure, and its sensitivity [2 μg/L (0.3 nmol/L) in 0.05 mL of sample] was more than sufficient to measure OC in human serum or bone samples. The assay is calcium-dependent, as could be expected from a protein with a calcium-dependent configuration (1). Others have therefore also included either EDTA or calcium in the assay buffer to exclude interference from the variable calcium content of the samples; however, not all antisera are calcium sensitive, especially when they recognize the carboxy-terminal part of the protein (18). The cross-reaction of the anti-hOC guinea pig antiserum was minimal with OC from species with large differences in the structure of this protein (Figure 2); it was best with ovine OC but not completely parallel with bovine OC. The antigenic recognition site(s) of our antiserum could not be clearly defined because several fragments were not recognized, although these combined fragments span the full length of intact OC. The probable epitope is therefore either a small region overlapping the fragments used or a configurational calcium-sensitive region.

Most previous RIAs or enzyme-linked immunoassays of OC had a heterologous assay configuration; the apparent OC concentration thus measured in human serum was reported as being much lower than in most animal sera. The present homologous RIA of hOC, however, recognizes much higher serum OC concentrations than the heterologous assays. We therefore carefully calibrated the hOC standard by measuring its absorptivity and determining its amino acid content. A previous human homologous assay also found higher OC concentrations (21, 23), whereas an assay based on antisera raised against a synthetic carboxyl-terminal fragment of OC (identical in human, ovine, and bovine species) also found higher than usual OC concentrations: 2.6 nmol/L, or 15 μg/L in adults (30). Despite the higher concentration detected in human serum, excellent correlations were observed between values obtained with the homologous and heterologous assays (Figure 6). This is further documented by the relative agreement of the OC content of coded samples used for an interlaboratory comparison (Figure 8). It may therefore well be that the real OC content of human serum is closer to that of other species than was previously thought.

Serum OC in healthy subjects is highly age-dependent, with values in children being several-fold higher than in adults (31–33; and Figure 5). Peak concentrations of OC were similar in girls and boys but occurred at an earlier age in girls, as could be expected from their earlier growth spurt. After puberty, a slow decline towards adult concentrations was observed, plateauing earlier in women than in men (Figure 5). After age 25, no further changes in mean serum concentrations of OC were observed in men. Serum OC was higher in healthy men than in premenopausal women, as reported before (5, 34, 35). Also as observed previously, postmenopausal women showed a significant increase in hOC (1–3), without significant differences between normal, osteoporotic, or osteoarthritic women.

In samples from patients with diseases known to increase bone turnover, the expected increase of OC was observed (Paget disease, hyperparathyroidism, and hyperthyroidism), whereas decreased values were observed in cases of mild vitamin D deficiency or glucocorticoid excess (1–3).

In early pregnancy the serum OC concentration was decreased, but gradually increased towards normal at the end of human pregnancy, as was observed previously with heterologous assays (36, 37). A similar trend of serum OC concentration during pregnancy, but with differences in absolute concentrations, was found when the same samples were measured with a bovine OC RIA (38).

The increase in serum OC in patients with predialysis chronic renal failure is probably due to several phenomena: secondary hyperparathyroidism with concomitant increased bone turnover and accumulation of OC fragments (39–41). This can also be derived from a steeper slope in the correlation between OC values measured with the human and bovine OC assays (Figure 6).

The results of the present homologous hOC assay thus largely confirm and extend previous data obtained for healthy subjects and for patients with diseases of calcium metabolism (1–4, 24). The absolute concentration of OC in human serum, however, seems to be much higher than previously estimated from measurements in heterologous assays. The interspecies difference in serum OC is therefore also less than previously suspected.

The help of G. Massa, M. Vanderschueren-Lodeweyckx, J. Knops, and many clinicians of the Hospitals of the Catholic University Leuven and the Vlaamse Krui in the collection of sera is greatly appreciated. The study was supported by grant no. 3.0044.89 from the Belgian FGWO.

References