Development and Evaluation of Three Immunofluorometric Assays That Measure Different Forms of Osteocalcin in Serum

SANNA-MARIA KÄKÖNEN, 1* JUKKA HELLMAN, 2 MATTI KARP, 1 PIRJO LAAKSONEN, 1 KARL J. OBRANT, 3 H. KALEervo VÄÄNÄNEN, 4 TIMO Lövgren, 1 and KIM PETTERSSON 1

Background: Circulating human osteocalcin (hOC) has been used as a marker of bone formation. Our aim was to validate three immunofluorometric assays (IFMAs), measuring different forms of hOC.

Methods: The two-site IFMAs were based on previously characterized monoclonal antibodies. Assay 2 recognized intact hOC, assays 4 and 9 measured the NH2-terminal mid-fragment and the intact hOC. In addition, assay 9 required hOC to be γ-carboxylated.

Results: A 76–79% increase of serum immunoreactive hOC was found in the postmenopausal group compared with the premenopausal group with all IFMAs. With EDTA-plasma samples, the observed increases were lower (49–65%). The hOC concentration in the postmenopausal group receiving hormone replacement therapy was 42–44% lower than that in the postmenopausal control group in both serum and EDTA-plasma samples. The depressed carboxylation in warfarin-treated patients was accompanied by lower results in assay 9. The ratio of assay 9 to assay 4 totally discriminated the warfarin-treated patients from the controls. Assay 9 showed the smallest decreases in measured hOC after storage of serum or plasma for 4 weeks at 4 °C, followed by assay 4 and assay 2. Results from the last assay were <17% of their initial values after 4 weeks of storage. No diurnal variation was observed with assay 9 as opposed to the two other IFMAs.

Conclusion: The three assays with their distinct specificity profiles (intact vs fragmented and carboxylated vs decarboxylated hOC) may provide valuable tools for investigating the significance of different hOC forms in various bone-related diseases.

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Human osteocalcin (hOC), 5 also designated bone Gla protein, is an abundant noncollagenous protein synthesized by osteoblasts (1). Although most of the synthesized hOC is bound to bone hydroxyapatite, a small part of it leaks into the blood stream (2). hOC concentrations in the circulation have been used in clinical investigations as a marker of bone formation (3). However, the discordant results obtained with different hOC assays have hindered widespread usage of hOC in clinical applications (4–7). Even when the same calibration preparation was used, hOC concentrations measured with different assays could not be directly compared because of different circulating forms of hOC (6, 8). Proteolysis in vivo generates fragments, especially the large N-terminal mid-fragment, which is the main product of hOC breakdown in the serum (9). In addition, the in vitro instability of hOC in serum or plasma samples after venipuncture presents a complication (10, 11). In addition, incomplete γ-carboxylation of hOC has been indicated by Poser et al. (1). When Ca2+ binds to the γ-carboxylated glutamic acid residues in hOC, an α-helix structure is formed that affects the conformation (12, 13). hOC can also interact with other Ca2+-binding proteins or complexes in circulation (12).

In this report, we describe three hOC assays with...
different specificities for circulating hOC forms. The detailed characterization of the monoclonal antibodies (Mabs) used has been reported previously (14). We here report the validation of the assays using samples from pre-, peri-, and postmenopausal women as well as postmenopausal women receiving hormone replacement therapy (HRT). A panel of warfarin-treated patients was also included. The correlation to commercially available hOC assays is described. We also report on the in vitro stability and diurnal variation of the hOC immunoreactivity as measured by the three assays.

Materials and Methods

CHARACTERISTICS OF MABs, IMMUNOASSAY PROCEDURES, AND EVALUATION

Three Mab-Mab combinations were selected on the basis of a previous study (14). Assay 2 is specific for the intact hOC, whereas assays 4 and 9 also detect the large NH$_2$-terminal fragment. In addition, assay 9 is relatively specific to the carboxylated form of hOC. The Mabs were biotinylated and europium-labeled as described previously, and hOC purified from femurs was used as a calibrator (14).

Samples and calibrators (10 μL of each) were added into wells of streptavidin-coated microtiter plates (Wallac Oy), followed by a mixture of biotinylated and Eu$^{3+}$-labeled Mabs in 50 μL of Delfia$^{®}$ Assay Buffer (Wallac Oy). For all three assays, 200 ng/well of capture Mab was used; 200 ng/well of the appropriate tracer Mab was used for assay 4, and 100 ng/well was used for assays 2 and 9. In assays 4 and 9, the Delfia Assay Buffer contained 5 mmol/L EDTA. After shaking at 35 °C for 1 h, the plates were washed, and the fluorescence was measured as described previously (14).

The between- and within-assay reproducibility, precision profile, and analytical detection limit were determined for each assay. Studies of serum sample linearity and recovery of calibrator antigen were performed. In the long-term stability test, serum, EDTA plasma, and lithium heparin samples from six in-house volunteers were collected and stored at –70°C, whereas assays 4 and 9 also detect the large NH$_2$-terminal fragment. In addition, assay 9 is relatively specific to the carboxylated form of hOC. The Mabs were biotinylated and europium-labeled as described previously, and hOC purified from femurs was used as a calibrator (14).

The correlations to commercially available hOC assays are described. We also report on the in vitro stability and diurnal variation of the hOC immunoreactivity as measured by the three assays.

Subjects and Blood Collection

Blood sample collections were in accordance with the Helsinki Declaration of 1975 as revised in 1996. Serum and EDTA-plasma samples were taken from 91 apparently healthy women, were allowed to stand for 30 min at room temperature, and then were centrifuged at 3000g for 20 min. The samples were aliquoted within 1 h and stored at –70°C. The women were divided into premenopausal (42 ± 4 years; n = 47), perimenopausal (48 ± 3 years; n = 11), and postmenopausal (56 ± 5 years; n = 31) groups according to menstrual status, based on questionnaire responses, and follicle-stimulating hormone concentration in serum samples measured by Delfia hFSH assay (Wallac Oy). The postmenopausal group was further divided into subjects receiving (56 ± 7 years; n = 16) or not receiving (56 ± 3 years; n = 15) HRT. All HRT patients had received treatment for at least 6 months.

The response to treatment affecting γ-carboxylation of hOC was studied using a cohort of 37 patients receiving warfarin treatment (19 women and 18 men; 73 ± 8 years) and 30 untreated controls (12 women and 18 men; 72 ± 6 years). Warfarin was taken for recurrent thromboembolism or chronic heart disease for at least 12 months, and the weekly dosage varied between 8.75 and 78.75 mg (mean, 33.6 mg). All but one of the patients had therapeutic prothrombin concentrations with an international normalized ratio of 2.0–3.8 as measured by the Prothrombin complex test (Diagnostic Stago). Detailed information of this panel has been reported by Obrant et al. (15).

For diurnal variation, samples were taken from six healthy premenopausal women who worked at the University of Oulu, Finland, at 0600 (0609 ± 021, mean ± SD), at 1200 (1207 ± 012), at 1800 (1819 ± 024), and at 2400 (2402 ± 011); hOC concentrations in these samples were measured with assays 2, 4, and 9.

Commercial Osteocalcin Assays

A subset of samples from the pre- (n = 15) and postmenopausal (n = 15) groups and the postmenopausal group receiving HRT (n = 6) were selected for a correlation study between two commercially available hOC two-site IRMAs: ELSA-OST-NAT and ELSA-OSTEO (CIS bio international) and the immunofluorometric assays (IFMAs) of this study.

Statistical Methods

The hOC values are given as mean ± SD. A weight of 5930 g/mol was used to calculate the molar concentrations of hOC. ANOVA followed by the Bonferroni test was used for subject groups in the panel of healthy controls. The Student t-test was used for comparisons of results from the warfarin panel, and repeated measures ANOVA was used for the stability and diurnal variation studies. Linear regression was used to analyze the relationship between two variable groups. P < 0.05 was considered significant.

Results

Analytical Characteristics of the Assays

The precision profiles were obtained from 12 replicates of each calibrator of each assay (Fig. 1). The detection limits, defined as the concentration corresponding to the mean value of 12 determinations of the zero calibrator + 2 SD, were 0.01, 0.008, and 0.012 nmol/L for IFMAs 2, 4, and 9, respectively. The within- and between-assay variations were calculated with three serum samples (mean concentrations, 0.81, 3.30, and 5.28 nmol/L, respectively, as measured with assay 2). The within-assay CVs were <5%
and the between-assay CVs (n = 12) were <8% for each of the assays. The linearity of the assays was estimated by two-, four-, and eightfold dilutions of five serum samples with the zero calibrator (Fig. 2). The observed increase in intact hOC may be caused by non-specific matrix effects or decreased proteolysis of hOC in samples diluted with the bovine serum albumin-containing diluent used for the calibrators. The analytical recoveries of hOC calibrator (1.69, 3.37, and 8.43 nmol/L) added to human sera (n = 9) were 92% ± 8.2%, 91% ± 5.3%, and 95% ± 4.6% for IFMAs 2, 4, and 9, respectively.

The results of long-term stability studies of hOC in serum, EDTA-plasma, or lithium heparin-plasma samples are shown in Table 1. In addition to poor stability of hOC measured by assay 2, there was a remarkable variation between individual samples after long-term storage. The concentration of total (assay 4) and γ-carboxylated (assay 9) hOC was unchanged or slightly increased during 4 weeks of storage at 4 °C. However, during storage at 22 or 35 °C, the stability of immunoreactive hOC as determined by assay 9 was superior to that of the total hOC assay (assay 4). The concentration of intact hOC was significantly decreased in serum (P ≤ 0.037) and heparin-plasma (P = 0.0049) samples after one cycle of freezing and thawing. In EDTA-plasma samples, this reduction was observed after four cycles (P = 0.017). Significant reduction of hOC immunoreactivity was observed after four

![Fig. 1. Dose–response curves of assays 2 (●), 4 (○), and 9 (▲) given as fluorescence counts vs concentrations of the hOC calibrator over the range 0.017–13.5 nmol/L, and the corresponding within-assay CVs (open symbols) calculated from 12 replicates.](image)

![Fig. 2. Dilution linearity of the three IFMAs tested by two-, four-, and eightfold dilutions of five serum samples into the diluent used for the calibrator. The results after the dilutions are given as mean percentages of the expected values ± SD. The hOC concentrations in the undiluted serum samples were 0.57–2.61 nmol/L as measured by IFMA 2. Filled symbols indicate that the dilution result was significantly different (P < 0.05) from the expected value.](image)
CLINICAL PERFORMANCE OF THE ASSAYS WITH HEALTHY SUBJECTS

The mean hOC concentrations (± SD) in serum samples as measured by IFMAs 2, 4, and 9 were 0.99 ± 0.29, 1.38 ± 0.37, and 1.21 ± 0.30 nmol/L in the premenopausal group (n = 47); 1.06 ± 0.29, 1.48 ± 0.39, and 1.31 ± 0.32 nmol/L in the perimenopausal group (n = 11); and 1.75 ± 0.61, 2.46 ± 0.81, and 2.17 ± 0.67 nmol/L in the postmenopausal group not receiving HRT (n = 16). On average, the hOC concentrations in the EDTA samples were slightly lower (5.4%, 7.8%, and 4.7%, as measured with assays 2, 4, and 9, respectively) than the concentrations measured in the serum samples of the same individuals. Compared with the premenopausal group, the serum hOC concentrations in the postmenopausal group were 76%, 77%, and 79% higher for assays 2, 4, and 9, respectively (P < 0.0001).

With EDTA-plasma samples, the differences were smaller (58%, 65%, and 49%, respectively). The mean hOC concentrations for both serum and EDTA plasma were 42-44% (P < 0.0001) lower in the group receiving HRT (n = 30) than in the postmenopausal group not receiving HRT. The perimenopausal and postmenopausal groups differed significantly from each other, but no significant differences between the pre- and perimenopausal groups were observed with either serum or EDTA-plasma samples. The correlation coefficients between assays 4 and 2 and between assays 4 and 9 were 0.97 (assay 4 = 1.346(assay 2) + 0.077; Syx = 0.156; and assay 4 = 1.147(assay 9) − 0.016; Syx = 0.144), and the correlation coefficient between assays 2 and 9 was 0.96 (assay 2 = 0.813(assay 9) − 0.013; Syx = 0.134) when samples from the healthy control panel (n = 89) were included. The corresponding values obtained with the EDTA-plasma samples were 0.96 [assay 4 EDTA = 1.247(assay 2 EDTA) + 0.138; Syx = 0.165], 0.97 [assay 4 EDTA = 1.194(assay 9 EDTA) − 0.199; Syx = 0.140], and 0.92 [assay 2 EDTA = 0.87(assay 9 EDTA) − 0.9; Syx = 0.166].

The performance of the IFMAs was also compared to two commercially available assays. The hOC concentration was 1.67 ± 0.42 nmol/L (n = 15) in the premenopausal group and 2.6 ± 0.89 nmol/L (n = 15) in the postmenopausal group as measured with ELSA-OSTEO-NAT. The corresponding values measured by ELSA-OSTEO assay were 2.77 ± 0.66 nmol/L (n = 15) and 4.91 ± 1.5 nmol/L (n = 15), respectively. The hOC concentration obtained in the postmenopausal group was 57% higher with ELSA-OST-NAT (P = 0.0009) and 79% higher with ELSA-OSTEO (P < 0.0001), and the values were decreased in the HRT group (n = 6) by 38% (P = 0.0048) and 40% (P = 0.0006), respectively. The regressions between IFMAs and IRMAs were all highly significant (P < 0.0001), but clear differences in slopes were observed [assay 2 = 0.662(ELSA-OST-NAT) − 0.074; r = 0.93; Syx = 0.212; assay 4 = 0.51(ELSA-OSTEO) − 0.073; r = 0.99; Syx = 0.136; and assay 9 = 0.428(ELSA-OSTEO) + 0.027; r = 0.96; Syx = 0.195].

Significant diurnal variation was observed in the hOC concentrations measured with the intact hOC-specific assay (assay 2) and with the total hOC-specific assay (assay 4), whereas no significant variation was measured by assay 9 (Fig. 3).

WARFARIN PANEL

The concentration of hOC as measured with assay 9 was lower in the warfarin-treated patients (0.83 ± 0.64

Fig. 3. Diurnal variation of immunoreactive hOC as measured by the IFMAs 2 (squares), 4 (circles), and 9 (triangles). Point of times indicated with filled symbols are significantly different (P < 0.05) from the basal values.

Fig. 4. The carboxylated/total hOC (#9/#4) and carboxylated/intact hOC (#9/#2) concentration ratios in warfarin-treated (n = 37) and control (n = 30) subjects. The horizontal lines of the box plots represent the 10th, 25th, 50th, 75th, and 90th percentiles.
nmol/L; n = 37) than in the control patients (2.2 ± 1.6 nmol/L; n = 30; P < 0.0001). There were no significant differences between the warfarin-treated patients and the controls in the amount of total hOC measured by assay 4 (1.59 ± 1.5 vs 2.14 ± 1.79 nmol/L; P = 0.173) or full-length hOC measured by assay 2 (1.25 ± 1.33 vs 1.31 ± 0.86 nmol/L; P = 0.816). The proportion of carboxylated hOC/total hOC was lower in the warfarin-treated patients compared with the controls (P < 0.0001) and a single cutoff ratio of 0.8 separated the two patient groups (Fig. 4). In addition, a significant (P < 0.0001) difference was observed between the carboxylated hOC/full-length hOC ratios for the two groups.

Discussion

In Vitro Stability of the hOC Forms

The instability of hOC has been reported to be method-dependent and related to preanalytical variables (10, 16, 17). Repeated freezing and thawing should be avoided, although the stability of the intact hOC in EDTA-plasma samples appears to be better in agreement with earlier reports (17). In hemolyzed samples, metalloproteases released by erythrocytes have been reported to cleave the intact hOC into smaller fragments (18). We were unable to demonstrate the stabilizing effect of heparin plasma compared with serum or EDTA plasma in long-term stability studies that was suggested in a previous report (16). In agreement with previous studies (19–21), lower hOC values were obtained for EDTA-plasma samples than for serum samples. Thiede et al. (22) have suggested that the higher amount of hOC in serum than in plasma samples is a result of hOC released from platelets during blood clotting. In the present study, the relative increase of hOC in serum compared with plasma was similar over the whole hOC concentration range, making it unlikely that the difference was attributable to release of hOC from platelets.

Clinical Performance of IFMAs

Despite the distinct specificity profiles of the three assays, their performance in measuring hOC in the menopausal sample panel was highly similar. The observed increase of serum hOC during the change in menopausal status is consistent with results of other studies (8, 23, 24). The hOC concentrations obtained with the ELISA-OST-NAT and ELSA-OSTEO assays were higher than the results obtained with the IFMAs. Differences in assay design or hOC calibrator preparations may be contributing factors. Reductions of 53% ± 5% (n = 4) and 56% ± 2% (n = 4) were observed when ELISA-OST-NAT calibrators were measured with IFMA 2 and ELSA-OSTEO calibrators were measured with IFMA 4, respectively, compared with the nominal values given by the manufacturer.

The diurnal rhythm of hOC has important implications for the selection of sampling times, e.g., in the monitoring of hOC changes after various pharmaceutical interventions. In agreement with previous reports, the hOC concentration was high at night and early morning and lower during the late morning and afternoon (25–27). Significant variations in the hOC concentration were observed with IFMAs 2 and 4. A similar but nonsignificant trend was seen with assay 9. This could explain the differences between the reported peak and nadir values (10–50%) with different hOC immunoassays (25–27).

The Clinical Significance of γ-Carboxylated hOC

Osteocalcin binds, through its γ-carboxylated glutamic acid residues, to hydroxyapatite, which has been used to distinguish the fully γ-carboxylated form from the undercarboxylated (uchOC) forms (2). The hydroxyapatite-binding capacity of circulating hOC is abnormally low in the elderly (28). Szulc and co-workers (29, 30) have shown that circulating uchOC is a marker of hip fracture risk in a population of institutionalized women and that the bone mineral density is decreased in women with increased uchOC. Separation of the hOC forms using hydroxyapatite is difficult because of inconsistent binding (29, 31).

Vergnaud et al. (32) have developed an ELISA for uchOC based on Mabs with low cross-reactivity to carboxylated hOC. They showed that uchOC predicted hip fracture risk independently of bone mass in elderly women. However, the uchOC concentrations measured with the same assay were not statistically different between the warfarin-treated patients and control subjects, whereas the ratio of uchOC to intact hOC differed significantly between the two groups (31). In our study, the concentration of γ-carboxylated hOC was significantly lower in warfarin-treated patients compared with controls (P < 0.0001). The ratio of γ-carboxylated to total hOC fully distinguished patients on long-term warfarin treatment from age-matched controls. We recently reported that the ratio of γ-carboxylated hOC to total hOC as measured with IFMAs 9 and 4, respectively, predicts the occurrence of fractures in older community-dwelling adults [Luukinen et al., submitted for publication; and Ref. (33)].

The described three hOC IFMAs show clear differences in their recognition of various circulating hOC forms. These assays are suitable for monitoring circulating hOC in several situations affecting bone metabolism.

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References


