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Immunoradiometric Assay for Intact Human Osteocalcin(1–49) without Cross-Reactivity to Breakdown Products

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Background: **Osteocalcin (Oc), a serum marker of bone turnover, circulates in several forms. We developed an assay for intact human Oc and investigated its clinical features.**

Methods: **We generated goat antibodies and N- and C-terminal Oc. The former was used on solid phase (polystyrene beads), and the latter was used as the tracer in an IRMA.**

Results: **The assay was linear with no cross-reactivity to Oc(1–43), total imprecision (CV) of <10%, and recovery of 100% ± 10%. Assay values for intact Oc in EDTA plasma samples were unchanged at 18–25 °C for 6 h. Values for intact Oc in serum, EDTA plasma, and heparin plasma samples did not change after storage on ice for 8 h. Serum samples from patients with various conditions were stored at −70 or −135 °C for up to 5 years and yielded *z*-scores comparable to an Oc(1-43) IRMA for all conditions except for renal failure. In renal failure, the Oc(1–43) assay values were increased, whereas the intact assay values were in the reference interval.**

Conclusion: **Decreases in Oc assay values are inhibited by calcium chelation, and slowed by reduced temperatures. The described assay for intact Oc allows improved specificity for bone compared with an assay for Oc(1–43).**

Osteocalcin (Oc) is the most abundant noncollagenous protein in bone, comprising 1–2% of total protein *(1, 2)*. It is synthesized only by osteoblasts and odontoblasts, with a small amount released de novo into the circulation *(2)*. Oc contains glutamic acid residues at positions 17, 21, and 24 that can be incompletely carboxylated in humans *(3)*. The full-length Oc peptide contains 49 amino acids, with subsequent breakdown to fragments 1–19, 20–49, 20–43, 1–43, and 44–49 in the liver, kidney, and serum *(4)*. Oc has been shown to bind Ca^{2+}, and it binds strongly to hydroxyapatite *(1)*. Oc is chemotactic for a variety of cells *(5)* and thus may play a role in osteoclast recruitment *(6)*. Observation of a viable Oc knock-out mouse with stronger, more dense bones *(7)* supports this hypothesis, but the exact function of Oc in bone metabolism is still unclear.

The serum concentration of Oc been shown to correlate with the extent of metabolic bone diseases characterized by increased bone turnover *(8–13)*. Additionally, Oc can be a specific marker for bone formation when formation and resorption are not balanced *(10)*.

Several efforts have been made to improve the clinical interpretation of assay results through (*a*) the use of human Oc calibrators and anti-human Oc antibodies rather than bovine components, (*b*) the reporting of patient values as a ratio to healthy subject values in that assay, and (*c*) validation of assays to determine the primary sequences to which capture and tracer antibodies bind *(14)*. The extent to which immunoreactivity is affected by the tertiary structure of Oc is an important difference among existing Oc assays *(15)*. The tertiary structure of Oc has been proposed to contain two calcium-binding α-helices surrounding a disulfide-stabilized β-turn. Lack of the disulfide bond between Cys^{23}-Cys^{29} or a decarboxylation of γ-carboxy-glutamic acid has been shown to disrupt the tertiary structure of Oc *(16)*. If changes in tertiary structure affect antibody affinity, immunoassay results may be affected by heterogeneous

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carboxylation or by the introduction of protease inhibitors that themselves bind calcium. In addition, some groups have reported that the freeze-thaw stability of Oc varies, whether in serum or buffer *(17)*, suggesting that the immunoreactivity of antibodies can be affected by other physical factors.

We therefore explored these issues as we analytically validated an assay for intact Oc assay. After the analytical validation, we examined samples from clinically characterized patients to evaluate the potential of the assay for clinical application.

Materials and Methods

ANTIBODY PURIFICATION

Goats immunized with Oc peptides were plasmapheresed. The antibodies were precipitated with saturated ammonium sulfate at a final concentration of 184 g/L. The pellet was resuspended in and dialyzed against phosphate-buffered saline. The antibody was affinity-purified on an agarose bead column conjugated with synthetic N-terminal Oc peptide for the capture antibody and a synthetic C-terminal Oc peptide for the reporter antibody.

PREPARATION OF THE SOLID PHASE

Rabbit α-goat antibody was adsorbed to a polystyrene bead (Hoover Precision), with the N-terminally directed antibody captured by the rabbit α-goat antibody and the beads blocked with normal horse serum to prevent nonspecific binding.

PREPARATION OF THE REPORTER ANTIBODY

The goat anti-Oc(43–49) was radiolabeled with ^{125}I using the chloramine T method. Briefly, tyrosine residues were activated using chloramine T oxidation in the presence of ^{125}I. The chloramine T was quenched with an excess of sodium metabisulfite. Free ^{125}I was separated from labeled antibody by size-exclusion HPLC.

CALIBRATORS AND CONTROLS

Oc(1–49) purified from human bone was sent blindly to two independent laboratories for quantitative amino acid analysis. The results obtained agreed within 4%. Antigen vials were assigned a concentration based on this quantitative amino acid analysis, and calibrators were prepared in 0.05 mol/L borate containing 5 g/L bovine serum albumin. The controls were built from the same antigen stock, but the control stock and dilution to control concentration were made in 0.05 mol/L borate containing 100 mL/L normal human serum.

WASH BUFFER

The wash buffer was 5 mL/L Tween 80 in 0.1 mol/L phosphate-buffered saline.

ASSAY PROCEDURE

Calibrator, control, or unknown samples (20 μL) were pipetted into the bottoms of borosilicate or polystyrene tubes. ^{125}I-labeled anti-Oc(43–49) antibody (300 μL) was added to all test tubes. The test tubes were vortex-mixed, and beads were dispensed into each tube. The tubes were incubated for 2 h at 18–25 °C and 190 ± 10 rpm (2.0-cm diameter orbit shaker), and then aspirated and washed three times with assay wash buffer. Test tubes were counted in a gamma counter. Radioactivity was directly proportional to the concentration of Oc. The radioactivity (cpm) of the calibrators was used to construct the calibration curve using a spline-smoothed curve.

SPECIFICITY AND SUPPRESSION OF ASSAY BINDING

Oc peptides were added to the assay E calibrator (200 μg/L), and these samples were assayed as unknowns. Reduced cpm in the samples with added Oc fragments compared with a control E calibrator indicated that the fragment was bound by one of the component antibodies of the reported assay.

IMPRECISION

The interassay CV was assessed with three pools of with below (hypo), within (normal), and above (hyper) the estimated health-related reference interval.

PATIENT POPULATION FOR DILUTION STUDY

Sera were collected over a 2-week period, during routine testing, from seven children (age range, 7–14 years) with chronic renal insufficiency from either glomerular (n = 2) or tubulo-interstitial (n = 5) diseases in whom the residual renal function was 25–70% of normal. An additional 15 sera were collected over a 3-week period, during routine testing, from children (age range, 6–18 years) referred to the clinical practice of one of the investigators (C.B.L.) for suspected disorders of bone metabolism, but in whom no pathologic process was identified subsequently. Samples were stored at −20 °C until assayed, and they were thawed and stored on ice during the assay. Samples were refrozen within 60 min, and no samples were frozen and thawed more than twice. The samples from adults with renal failure were collected before routine hemodialysis and had increased parathyroid hormone; all of these adult samples were obtained anonymously from the Minneapolis regional kidney dialysis program with informed consent.

DILUTION LINEARITY

Samples from children with renal insufficiency were used to validate dilution linearity. These samples were chosen for the high content of Oc and Oc breakdown products. The clinical samples were all diluted with calibrator matrix, and all dilutions were serial dilutions.

Z-SCORE ANALYSIS OF PATIENT POPULATION

For evaluation of the assay, fasting state serum samples were obtained from healthy young women [n = 30; age, 32.0 ± 2.8 years (mean ± SD)]; healthy elderly women (n = 30; age, 74.2 ± 3.4 years); estrogen-treated postmeno-

pausal women (n = 15; age, 73.5 ± 3.6 years); hypoparathyroid subjects (n = 5; age, 61.6 ± 12.9 years); untreated osteoporotic women (n = 15; age, 65.0 ± 4.5 years); patients with Paget disease (n = 5; age, 75.4 ± 13.8 years); hyperparathyroid subjects (n = 5; age, 54.6 ± 16.8 years); and patients with renal failure (n = 5; age, 66.4 ± 6.1 years). All of these samples were obtained from the Mayo Clinic with informed consent.

comparison of *z*-scores

Two immunoassays for Oc from CIS Biointernational were compared side by side with the reported assay. The CIS-ELSA-OSTEO assay measures the fragment Oc(1-43) in addition to the intact molecule. The CIS-ELSA-OST-NAT assay measures intact Oc exclusively. The healthy young and elderly women had bone mineral density values within 2 SD of the age-matched mean, no history of fracture, and were not on medication known to affect calcium metabolism. The disease subjects were all classified using standard clinical criteria.

sample stability analysis

Samples were obtained from healthy male and female donors 21–60 of age from the DiaSorin Inc., employee population with informed consent. Multiple EDTA plasma, heparin plasma, serum separator, and serum tubes were collected. To obtain t = 0 samples, one EDTA whole blood and one heparin whole blood aliquot was centrifuged in a 4 °C Eppendorf 5414 microcentrifuge to separate cellular material. The plasmas were aspirated and stored at −70 °C for 13 samples within 10 min of drawing and within 15 min for all samples. Because of the difficulty of obtaining and freezing serum within 10–15 min and the parallel nature of degradation between heparin plasma and serum, the heparin plasma t = 0 sample value was used as the t = 0 sample value to model degradation in the serum stability analysis.

The remaining EDTA whole blood and heparin whole blood tubes were allowed to settle for 60 ± 20 min at 2–8 °C and 18–25 °C before centrifugation at 760*g* and collection of plasma. Samples were centrifuged at the temperature designated for that experimental condition. All plasmas were separated by the 2 h time point, and subsequent incubation occurred without cellular material present. The serum was allowed to clot at 18–25 °C for 30 ± 10 min, then was stored at 2–8 °C and 18–25 °C for an additional 30 ± 15 min before centrifugation at the appropriate temperature for that experimental condition. Serum was separated by the 2 h time point, and subsequent incubation occurred without cellular material present.

Results

analytical characteristics

The initial E calibrator binding was 21–30% of total counts at the beginning of tracer life, with typical values of ~25% (n = 10). At 10 weeks postiodination, the E calibrator binding was 12–19%, with typical values near 15% (n = 10). Nonspecific binding was <0.35% of total counts and often <0.2%. The logarithm of concentration was related to the logarithm of the cpm, as is characteristic of IRMA methodologies. We used an unweighted spline-smoothed curve fit, and the nonspecific binding was not subtracted from the calibrator cpm.

The limit of detection (2 SD above the value for nonspecific binding) in three separate assays with three lots of tracer at 8 days postiodination was 0.03–0.13 μg/L, with a mean (± SD) of 0.07 ± 0.05 μg/L. The reported limit of detection for the assay is 0.2 μg/L.

imprecision

Samples were run as duplicates in 10 different assays by six different technicians over 20 days. The interassay imprecision (CV) was 7.1–9.5%. The mean recovery of Oc at 10, 30, 60, and 110 μg/L added to patient sera, assayed in duplicate in three assays by two technicians, was 90–110%.

Linearity was evaluated with serial dilutions with the zero calibrators of sera from pediatric (n = 10) and adult (n = 12) renal patients. Renal samples were chosen because they contain high concentrations of both Oc and Oc breakdown products. The undiluted values ranged from 3.4 to 72 μg/L. The geometric mean values of observed/expected results at dilutions of 1:2, 1:4, and 1:8 were 104%, 104%, and 106%, respectively.

Synthetic human Oc(1–43, $GLA^{21,24}$) produced no detectable cross-reactivity up to 11 000 μg/L. The lack of suppression of the Oc(9–16) and Oc(30–43) peptides and the suppression of the Oc(1–16), Oc(37–49), and Oc(30–49), shown in Fig. 1, demonstrated that the capture antibody binds primarily to Oc(1–10) and the tracer binds in the region of Oc(43–49). The assay recognized (1–49, $GLA^{17,21,24}$), (1–49, $GLA^{21,24}$), and (1–49, $GLU^{17,21,24}$) equally (data not shown).

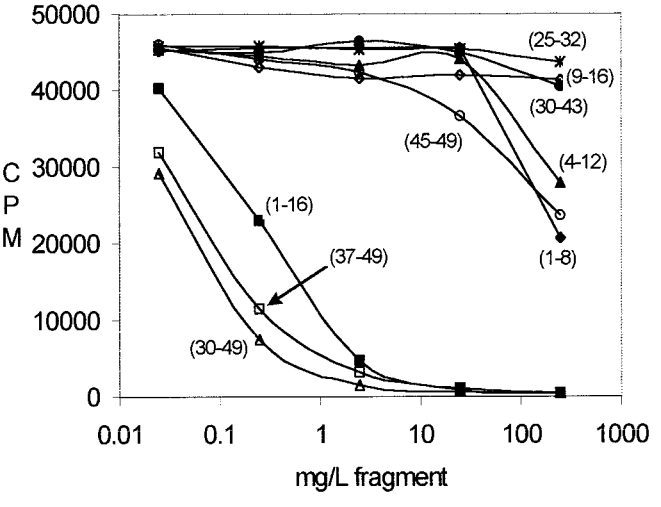

Fig. 1. Suppression of binding when synthetic Oc fragments were added to calibrator matrix containing 200 μg/L intact Oc.

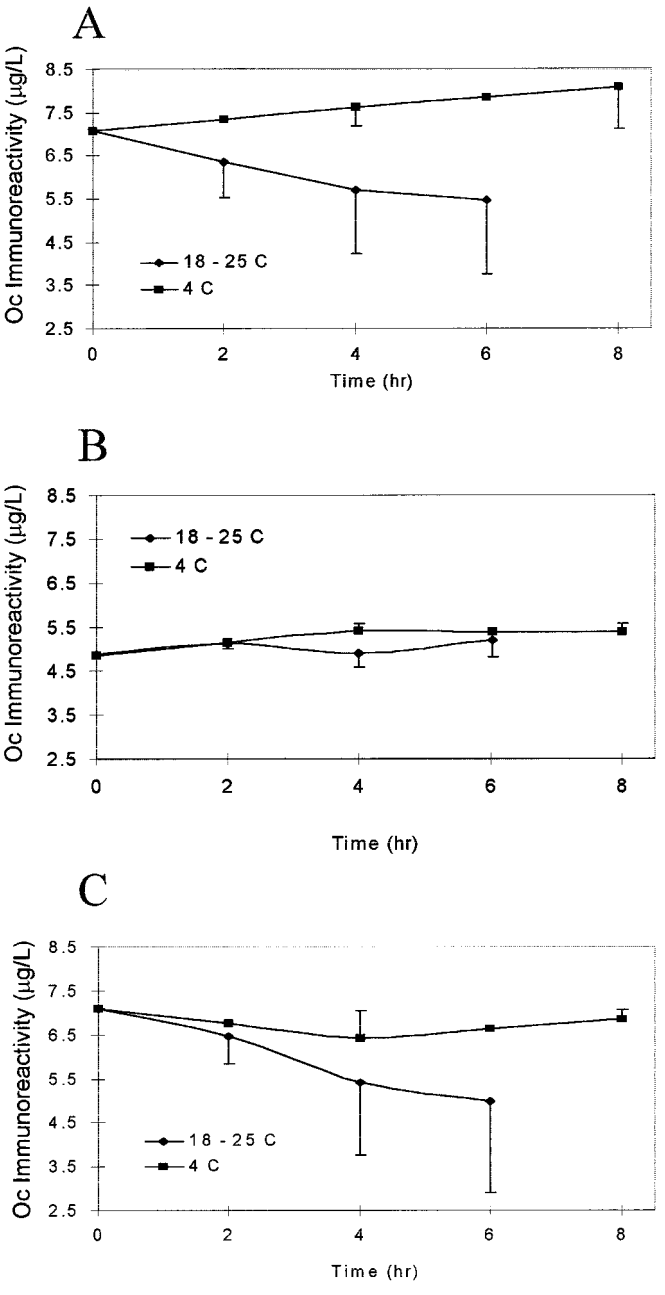

Fig. 2. Observed immunoreactivity in serum (*A*), EDTA plasma (*B*), and heparin plasma (*C*) samples incubated at 4 °C and 18–22 °C.

Matched serum, EDTA plasma, and heparin plasma specimens from healthy subjects were assayed after storage in an ice bath (2–8 °C) for 8 h and at 18–25 °C for 6 h (Fig. 2). Storage on ice for up to 8 h had no effect on results for serum or plasma. A significant ($P < 0.0001$) change was observed after storage for 2 h for heparin plasma and serum at 18–25 °C, but the change was not significant even at 6 h for EDTA plasma. Values for matched serum and heparin plasma samples were not statistically different from each other during storage at 18–25 °C, and thus the rates of change of assay values were not different in heparin plasma compared with serum. Values for EDTA plasma samples were slightly lower than heparin plasma values at t = 0, which could be attributed to a modest calcium-dependent matrix effect. Addition of as little as 2 mmol/L EDTA to the calibrators decreased the cpm, suggesting that the EDTA directly or indirectly inhibits antibody binding in the described assay. No significant changes were observed over time in EDTA plasma values in this experiment; however, changes were observed in both heparin plasma and serum, suggesting that calcium could be involved in the observed decreases in the amount of immunoreactive Oc that could be assayed over time. Changes in plasma sample values were also explored in a separate condition where the intact cellular material was simply allowed to settle at 2–8 °C and 18–25 °C. We did not test samples with disrupted cellular materials. These additional conditions yielded no difference in degradation rates compared with separated plasma (data not shown). Repeated freeze-thaw cycles of plasma yielded sample value imprecision within the magnitude of the interassay precision.

z-SCORE ANALYSIS

The CIS ELSA-OSTEO Oc(1–43) assay and the two intact Oc(1–49) assays had dissimilar absolute means (Table 1), but had similar *z*-scores (Table 2) for most of the tested conditions. The two intact assays did not differ significantly in *z*-scores for any population tested. For patients with renal failure, the CIS ELSA-OSTEO Oc(1–43) assay *z*-score was significantly higher than that of the Oc(1–49) assays ($P < 0.0001$).

Table 1. Results for each method in eight clinical groups [mean (SD)].

	Young healthy women	Elderly healthy women	Estrogen-treated women	Hypopara-thyroidism	Osteoporosis	Paget disease	Primary hyperpara-thyroidism	Renal failure
Number of subjects	30	30	15	5	15	5	5	5
Age, years	32 (3)	74 (3)	74 (4)	62 (13)	65 (4)	75 (14)	55 (17)	66 (6)
CIS ELSA-OSTEO (1–43), µg/L	21.2 (5.3)	25.7 (8.1)	17.2 (7.4)	13.7 (7.8)	34.7 (20.0)	77.8 (22.2)	64.0 (34.5)	51.0 (17.3)
DiaSorin N-tact Osteo SP (1–49)®, µg/L	4.7 (1.6)	5.7 (2.2)	3.6 (2.0)	2.8 (1.6)	6.5 (3.1)	21.7 (8.1)	16.3 (10.5)	5.3 (1.5)
CIS ELSA-OST-NAT (1–49), µg/L	12.3 (3.2)	14.8 (4.5)	10.2 (4.4)	7.6 (4.0)	18.1 (8.2)	43.3 (12.4)	34.3 (18.4)	16.2 (5.0)

Table 2. Results[a] for each of three assays in eight clinical groups.

	Young healthy women	Elderly healthy women	Estrogen-treated women	Hypopara-thyroidism	Osteoporosis	Paget disease	Primary hyperpara-thyroidism	Renal failure
Number of subjects	30	30	15	5	15	5	5	5
Age, years	32 (3)	74 (3)	74 (4)	62 (13)	65 (4)	75 (14)	55 (17)	66 (6)
CIS ELSA-OSTEO (1–43)	0 (1)[b]	0.9 (1.5)	−0.8 (1.4)	−1.4 (1.5)	2.6 (3.7)	10.6 (4.2)	8.0 (6.5)	5.6 (3.3)
DiaSorin N-tact Osteo SP (1–49)	0 (1)[b]	0.6 (1.4)	−0.7 (1.3)	−1.3 (1.0)	1.2 (2.0)	10.9 (5.2)	7.4 (6.7)	0.37 (0.97)
CIS ELSA-OST-NAT (1–49)	0 (1)[b]	0.8 (1.4)	−0.7 (1.4)	−1.5 (1.2)	1.8 (2.5)	9.7 (3.9)	6.9 (5.7)	1.2 (1.4)

[a] Values are [mean (SD)] of the individual *z*-score results for each group, for each assay, with the indicated sample size relative to the reference population of young women.

[b] Values [mean (SD)] for the CIS ELSA-OSTEO, DiaSorin N-tact Osteo SP, and CIS-ELSA-OST-NAT in the young women were, respectively, 21.2 (5.3), 4.7 (1.6), and 12.3 (3.2) μg/L.

Discussion

Our study addresses the relative utility of intact Oc assays and assays for the Oc(1–43) fragment. Several studies *(15, 17, 18)* have reported greater sample stability with Oc(1–43) immunoassays compared with intact Oc assays, without showing that the clinical utility is compromised with reasonable methodology or showing that the stability concerns are eliminated by use of an Oc(1–43) assay.

The N-terminal Oc(1–43) fragment has been shown to circulate in vivo, but its origin is currently unknown. It has been reported in the supernatant of osteoblastic cells, but the study was performed without a control for protease activity in the tissue culture from the matrix itself or from the cells *(18)*. Therefore, it is not clear if some Oc(1–43) is a de novo product or if it is exclusively a breakdown product.

In the absence of convincing evidence that this Oc(1–43) fragment is produced by osteoblasts, it would seem intuitive that the preferred assay for osteoblast activity would be an intact Oc assay that does not measure fragments, the production of which is highly dependent on kidney function. In this retrospective investigation of the classification accuracy of intact Oc measurements of banked serum samples, there appeared to be no improved discrimination between healthy patients and disease states using an IRMA that also recognizes the Oc(1–43) fragment. However, in the renal failure samples, the Oc(1–43) assay values appear significantly increased, whereas the intact Oc values not. This suggests that an intact Oc IRMA without cross-reactivity to breakdown products has greater specificity to bone metabolism in cases of renal insufficiency. Moreover, as assayed by the intact assay, samples were stable under various collection and storage conditions for up to 8 h, and retrospective analysis of frozen serum samples stored up to 5 years provided expected results.

Oc immunoreactivity has repeatedly been shown to be affected by many natural and some artificial phenomena, with decreased immunoreactivity attributed to degradation of Oc. It was reported that protease inhibitors have protective effects on sample stability before freezing in one assay *(18)*, had no effect in the present assay, and decreased the observed immunoreactivity of a third assay during freeze-thaw cycles *(19)*. A control for possible matrix effects in the report of protective effects of a protease cocktail *(18)* was not reported, although one of the protease inhibitors used is known to bind calcium, which has been shown to affect the tertiary structure *(16)* and immunoreactivity of Oc in some assays *(15)*. One Oc assay that is said to measure Oc(1–43) as well as the intact molecule nonetheless has problems with sample stability *(20)*; the specificity of that assay must be questioned, however, on the basis of our earlier work *(21)*. Several assays that measure Oc(1–43) in addition to the intact molecule showed increased immunoreactivity in samples stored in liquid form, apparently because the immunoreactivity of a synthetic Oc(1–45) fragment was higher than that of native Oc *(15)*.

Because in vitro degradation of Oc clearly occurs and the reported conclusions vary with the immunoassays used to study it are contradictory, data should be interpreted with caution. Immunoassays can only suggest degradative events. Effects of matrix and tertiary structure must be explored thoroughly and reported. One constraint of the present assay is the reduced cpm caused by EDTA. Conclusions about Oc measurements will be more credible with these additional data. Because the reported immunoreactivity is dependent on so many variables, it appears necessary to assess apparent sample stability and clinical classification accuracy of each assay individually.

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