Undercarboxylated Osteocalcin and Development of a Method to Determine Vitamin K Status

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We developed a RIA involving a polyclonal antibody against bovine osteocalcin, which has a carboxy-terminal epitope. Although the antibody recognizes both native and decarboxy osteocalcin, the two forms of osteocalcin were differentiated by adsorption to barium sulfate, taking advantage of the calcium-binding properties of the vitamin K-dependent gla domain. To test the clinical application of undercarboxylated osteocalcin, we examined the effect of minidose warfarin on this measure in nine healthy subjects, ages 60 to 80 years. The percentage of undercarboxylated osteocalcin increased by 170% ± 36% (mean ± SE) after 7 days of treatment with warfarin, 1 mg/day. The effectiveness of undercarboxylated osteocalcin as a sensitive measure of vitamin K nutritional status was further established when concentrations dropped to 17% ± 14% below baseline with 2 days of repletion with vitamin K1, 5 mg/day, during which prothrombin times did not leave the normal range.

Indexing Terms: radioimmunoassay/anticoagulants/nutritional status/warfarin

Osteocalcin is a 49-residue, 5700-Da protein found in bone and dentin (1). Osteocalcin requires vitamin K for its synthesis, with the vitamin functioning as a cofactor for a microsomal carboxylase in the posttranslational synthesis of three specific glutamic acid residues to γ-carboxyglutamic acid (gla) (2). These gla residues allow the protein to bind to calcium and therefore also to hydroxyapatite. If sufficient vitamin K is not available because of a dietary deficiency or vitamin K antagonism by anticoagulants, the osteocalcin produced lacks some or all of the gla residues. The carboxylation state of osteocalcin, determined by the differential binding of osteocalcin and its undercarboxylated form to hydroxyapatite, has been shown to be responsive to changes in vitamin K status (3–12). In addition, an increase in undercarboxylated osteocalcin has been shown to be indicative of risk of hip fracture in elderly institutionalized women (13), which adds to the growing body of evidence suggesting a role for vitamin K in metabolic bone disease (14–21).

Use of mini- or low-dose regimens of warfarin, a vitamin K antagonist, is currently being investigated as a more efficacious treatment modality for managing recurrent thrombotic events (22). Traditional clotting assays, including prothrombin time, do not have adequate sensitivity to monitor these new regimens and thus new techniques have been introduced. Increases in undercarboxylated osteocalcin have been uniformly noted in studies of subjects on conventional anticoagulant regimens (4–9). However, the effect of minidose therapy has not been specifically studied. Therefore, to test the responsiveness of undercarboxylated osteocalcin in a clinical application, we examined the effect of minidose warfarin by using an RIA for osteocalcin and a modified approach for extraction of the undercarboxylated species. In addition, the usefulness of undercarboxylated osteocalcin as a marker of vitamin K status was further studied by repletion of the anticoagulated subjects with vitamin K1.

Materials and Methods

Purification of Osteocalcin

Bovine osteocalcin was purified by a modification of the method of Gundberg et al. (23). Briefly, 200 g of bovine cortical bone powder (COLLA-TEC, Plainsboro, NJ) were extracted with 0.5 mol/L EDTA for 17 h at 4 °C and, after centrifugation, dialyzed overnight against 20 mmol/L Tris (pH 7.8) with Spectrapor 1 membrane tubing (Spectrum, Houston, TX). The Tris buffer was replaced with deionized water and dialysis continued for 4 days with many water changes. The dialysate was lyophilized and then dissolved in 0.05 mol/L NH4CO3 and applied to a Sephadex G-75 column (Pharmacia, Piscataway, NJ) equilibrated with the same buffer. Fractions containing osteocalcin were pooled, lyophilized, dissolved in 0.07 mol/L NH4CO3, applied to a DEAE Sephadex: fast-flow column (Pharmacia), and then eluted with a 0.07–0.7 mol/L NH4CO3 gradient. Osteocalcin purity was assessed and identification was confirmed by amino acid analysis and sequencing of the first four residues by the Tufts Protein Chemistry Facility, and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with 20% slab gels.

Antiserum

Antibodies to the purified bovine osteocalcin were produced in New Zealand White rabbits by using a Freund's adjuvant regimen (24). The initial inoculation...
of a 50:50 mixture of osteocalcin complexed with polyvinylpyrrolidone in phosphate-buffered saline was emulsified with Freund's complete adjuvant (Pierce Chemical Co., Rockford, IL) to a final concentration of 0.67 g/L osteocalcin; 0.25 mL was injected subcutaneously at each of four sites. Subsequent injections at 3- to 4-week intervals included Freund's incomplete adjuvant. Blood was drawn from the central ear artery of the rabbits before the initial inoculation and 10 days after each injection.

Iodination

The purified bovine osteocalcin was iodinated by the Chloramine T procedure. The labeled protein was separated from the unreacted free iodine by gel filtration on a Sephadex G-25 column equilibrated with 0.02 mol/L Tris buffer, pH 8.0.

Immunoassay

The RIA was developed based on the methods of Gundberg et al. (23) and Delmas et al. (25). Dilutions were made in assay buffer containing 0.02 mol/L Tris, 0.15 mol/L NaCl, 2 mmol/L CaCl₂, 10 g/L bovine serum albumin, and 10 mL/L Triton X-100, pH 8.0. The assay mixture consisted of 100 μL of serum or plasma, or calibrator, 100 μL of 125I-labeled osteocalcin (10 000 cpm), 100 μL of nonimmune rabbit sera (Pel-Freez Biologicals, Rogers, AR) diluted 1:40, 100 μL of anti- serum at a final dilution of 1:10 500, and 125 μL of assay buffer. The components were vortex-mixed and incubated in polypropylene tubes for 24 h at 4 °C. The antiserum was precipitated with a 1-mL second antibody solution of 10 mL/L goat anti-rabbit IgG (Pel-Freez) and 25 g/L polyethylene glycol in 0.02 mol/L Tris buffer, pH 8.0. After a 2-h incubation at 4 °C the samples were centrifuged at 1500g, decanted, and counted for radioactivity for 1 min (Cobra II gamma counter; Packard Instrument Co., Meriden, CT). The intra- and interassay CVs for this assay are 4.9% and 6.8%, respectively.

Antigenic Site Determination

Epitope mapping of the osteocalcin antibody was performed with the SPOTs kit (Cambridge Research Biochemicals, Wilmington, DE) and by testing the cross-reactivity of specific synthetic peptides of the human osteocalcin molecule with the antibody by RIA. Peptides corresponding to amino acids 1–12, 12–32, and 36–49 were synthesized by the Protein Chemistry Facility at Tufts Medical School, and a peptide of amino acids 31–36 was a gift of the Millipore Corporation (Bedford, MA) and synthesized on a Millipore 9050 plus PepSynthesizer. Calcium dependence of the assay was examined by replacing the calcium in the assay buffers with 25 mmol/L EDTA.

Effect of EDTA, Sodium Heparin, and Sodium Citrate Anticoagulants

The effect of anticoagulants used in blood collection on osteocalcin concentration was examined in speci-
diseases, diabetes, or pregnancy. Protein C concentrations, measured in all subjects, were within the normal range. No subject was taking oral anticoagulants, used antiinflammatory medications including aspirin on a regular basis, or used antibiotics, anticonvulsants, barbiturates, or phenobarbital within the last 6 months. In addition, no subject was taking any drug known to influence the response to warfarin.

Subjects resided in the Metabolic Research Unit at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University for 14 days and consumed a marginally low vitamin K diet (40 μg/day) throughout the study. After a baseline period of 4 days, subjects received a low dose of warfarin, 1 mg/day (Coumadin®, Du Pont Pharmaceuticals, Wilmington, DE), for the next 7 days and were then repleted on days 12 and 13 with 5 mg of vitamin K1 (Mephyton®, Merck, West Point, PA). Adverse effects of warfarin were monitored by prothrombin and activated partial thromboplastin times (measured every other day), and subclinical vitamin K deficiency was assessed by urinary gla excretion (measured daily). If subjects fell outside the safety criterion of an abnormal clotting time or a urinary gla excretion that declined on any given day by >25% or decreased by 15% for 3 consecutive days compared with baseline values, they would be repleted with vitamin K. Twenty-four-hour urine specimens were collected daily throughout the study, and fasting blood specimens were obtained on days 1, 5, 6, 8, 10, 12, and 14. Prothrombin and activated partial thromboplastin times were performed on citrated plasma with an MLA Electra 800 automated clot timer (Medical Laboratory Automation, Pleasantville, NY) with Dade reagents (Baxter Healthcare Corp., Miami, FL). Serum for measuring osteocalcin and undercarboxylated osteocalcin was frozen at −70 °C until analysis, and all specimens from each individual were analyzed in the same run.

Statistics

Comparison of recoveries of osteocalcin-supplemented normal and warfarinized serum samples were analyzed with unpaired Student's t-test. Overall changes in osteocalcin, undercarboxylated osteocalcin, and prothrombin time were assessed by repeated-measures analysis of variance, whereas differences between individual time points were evaluated with paired Student's t-test. Similar statistical tests were carried out when examining the effects of anticoagulants used in blood collection on osteocalcin concentrations, with the exception of gender comparisons, which were analyzed with unpaired Student's t-test.

Results

Osteocalcin Assay Characterization

Analytical recoveries of added osteocalcin were compared in normal serum and in serum from subjects who were treated for 7 days with 1 mg/day of warfarin. Recoveries from the normal and warfarinized speci-

Table 1. Analytical recovery of osteocalcin added to normal and warfarinized serum.

<table>
<thead>
<tr>
<th>Osteocalcin added, μg</th>
<th>Normal serum</th>
<th>Warfarinized serum</th>
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<tbody>
<tr>
<td></td>
<td>Sample 1</td>
<td>Sample 2</td>
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<tr>
<td>1.0</td>
<td>112</td>
<td>90</td>
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<tr>
<td>5.2</td>
<td>101</td>
<td>94</td>
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<tr>
<td>11.2</td>
<td>105</td>
<td>104</td>
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<tr>
<td>18.0</td>
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mens were not statistically different (Table 1). As shown in Fig. 1, dilutions from these same specimens also showed similar linearity. The minimum detectable concentration for the RIA, defined as 2 SD from the zero calibrator ($B_0$), was 0.40 μg/L. The intra- and interassay CVs of the assay were 8.2% and 6.5% for a low osteocalcin concentration (5.7 μg/L) and 4.9% and 6.8% for an average concentration (9.8 μg/L), respectively.

The BaSO4 adsorption method presented here discriminates native and undercarboxylated osteocalcin on the basis of the differential divalent metal binding properties of the gla domain. Subsequent to adsorption, the immunoassay antibody must detect both native and undercarboxylated osteocalcin. This was examined by constructing typical assay calibration curves, with concentrations ranging from 0.78 to 50 μg/L, with native osteocalcin and decarboxylated osteocalcin. As shown in Fig. 2, decarboxylated osteocalcin cross-reacts in the assay, as evidenced by the superimposable curves. To further define the antibody epitope, we used the SPOTs kit, in which overlapping 8-amino-acid peptides were synthesized on a cellulose membrane and probed with the osteocalcin antibody; synthetic peptides of the human osteocalcin sequence were tested in the RIA. Results from the SPOTs kit indicated reactivity between the antibody and the region of the
osteocalcin molecule that includes amino acids 31 to 36. However, when a peptide of this region was synthesized and tested in the RIA, no cross-reactivity was observed (Fig. 3). Similarly, there was no cross-reactivity with peptides from amino acids 1 to 12 and 12 to 32, encompassing the amino-terminal and gla domain of the molecule. There was, however, cross-reactivity of a peptide encompassing the carboxyl-terminal portion of the molecule (36–49), although at ~1000 times the concentration. Results in the carboxyl-terminal region from the SPOTs kit were inconclusive because of nonspecific binding of the second antibody. When EDTA was added to the assay buffer to assess calcium dependence, the binding curves obtained were identical to those generated in the presence of calcium; however, the maximum binding was decreased 50%.

Comparison between osteocalcin measured in serum and plasma is shown in Fig. 4. There were no differences observed between osteocalcin measured in serum specimens and plasma specimens prepared with either EDTA or sodium citrate. However, osteocalcin concent-

![Graph](image1)

Fig. 2. RIA calibration curves comparing native and heat-decarboxylated bovine osteocalcin.

![Graph](image2)

Fig. 3. Cross-reactivity of synthetic human osteocalcin peptides (amino acids 1–12, 12–32, 31–36, and 36–49) in the osteocalcin RIA.

![Graph](image3)

Fig. 4. Percentage difference (mean ± SE) between osteocalcin measured in serum and osteocalcin measured in EDTA, sodium citrate, and sodium heparin plasma.

Only sodium heparin plasma specimens were significantly different from serum (P = 0.001). Specimens were obtained from 16 healthy fasting subjects (7 men, 9 women), ages 25 to 72.

Undercarboxylated Osteocalcin Assay

The concentration of barium sulfate providing the maximum amount of undercarboxylated osteocalcin for measurement as well as acceptable discrimination of carboxylated and undercarboxylated osteocalcin was chosen after testing native and decarboxylated osteocalcin with concentrations of barium sulfate ranging from 0.5 to 300 g/L. With a barium sulfate concentration of 100 g/L, >80% of undercarboxylated osteocalcin remains in the supernate after adsorption, whereas >95% of carboxylated osteocalcin remains bound (Fig. 5). Specimens from the nine subjects in the minidose warfarin study were analyzed for undercarboxylated osteocalcin upon entry to the study, after adsorption to both barium sulfate and hydroxyapatite. Concentrations measured after treatment with 100 g/L barium sulfate (1.74 ± 0.20 µg/L) were an average of 42% ± 15% (P < 0.05) higher those treated with 25 g/L hydroxyapatite (1.30 ± 0.19 µg/L).

Changes in Undercarboxylated Osteocalcin with Minidose Warfarin Administration

The response of undercarboxylated osteocalcin to antagonism with minidose warfarin therapy was examined in nine healthy subjects, ages 60 to 80 years. As shown in Fig. 6, there was a significant increase of 170% ± 36% (P < 0.01) in undercarboxylated osteocalcin, as compared with baseline (day 5), after 7 days of minidose warfarin administration (1 mg/day); i.e., values increased from 15% ± 4% to 38% ± 9%. After 2
days of repletion with 5 mg of vitamin K₁, values dropped rapidly to 10% ± 2%, concentrations that were an average of 17% ± 14% below baseline (P < 0.05). These changes can be attributed to the undercarboxylated portion of osteocalcin, there being no changes in total osteocalcin during the study period (Fig. 6). Throughout the study the prothrombin time did not leave the normal range, and there was no effect of warfarin or vitamin K₁ dosing as compared with baseline in these same subjects, although the values after the depletion and repletion phases (days 12 and 14) were significantly different (P = 0.005).

Discussion

Since the discovery that osteocalcin can be quantified in serum and (or) plasma (30), many different assays, which generate values differing fivefold (31), have been developed. Some of this variability may be due to the type of assay, species of osteocalcin used for assay components, or antibody type or specificity. Inherent in the antibody specificity is the antigenic site of osteocalcin recognized and the degree of calcium dependence. This specificity is important, given that immunoreactive fragments have been recognized in the serum and urine of chronic renal-failure patients (32), and fragments can be detected in the serum of normal subjects (29, 33). Garnero et al. (34) have estimated that the intact molecule comprises 36% of circulating osteocalcin in healthy subjects, whereas minor fragments contribute 14% and an N-terminal mid-fragment contributes 30%. Thus the potential for fragment generation by inadequate control over sampling conditions is another factor contributing to the wide variation in reference ranges. Despite these disparities in values, an interassay comparison with samples from patients with metabolic bone disease has suggested that results from different laboratories are comparable when expressed in relation to normal values (35). However, two subsequent reports (36, 37) have concluded that results are not comparable in most disease states, particularly in patients with renal failure.

To characterize this assay and validate its usefulness in measuring both carboxylated and undercarboxylated osteocalcin, we undertook to map the epitopes by using two approaches, both involving synthetic peptides. The fact that native osteocalcin and decarboxylated osteocalcin generated parallel and superimposable curves suggested that the antibody epitope was not located in the gla domain. This was confirmed by the absence of cross-reactivity with synthetic peptides in that region. The fact that there was also no cross-reactivity of peptides in the amino-terminal end of the

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**Fig. 5.** Amount of osteocalcin remaining in supernates after treatment with various amounts of barium sulfate. Osteocalcin-extracted serum was supplemented with either native (10.3 μg/L) or decarboxylated (8.3 μg/L) bovine osteocalcin.

**Fig. 6.** Response (mean ± SE) of percent undercarboxylated osteocalcin (A), total osteocalcin (B), and prothrombin time (C) to 7 days of treatment with 1 mg/day of warfarin (days 5 to 11) and 2 days of treatment with 5 mg/day of vitamin K₁ (days 12 and 13) in nine healthy subjects. Error bars for prothrombin time are too small to be visible.
molecule (1–12) suggests lack of an epitope in this region, although peptides of the human osteocalcin sequence were tested and the polyclonal antibody was raised against bovine osteocalcin. Results from the SPOTs kit suggested a possible mid-molecule epitope, although this was not confirmed with a synthetic peptide. Potential explanations for this disparity include faulty peptide synthesis in the kit, nonspecific binding, or conformation requirements. Assays with mid-molecule epitopes appear to have different characteristics, since the antibodies have all been raised against human osteocalcin and the epitope encompasses amino acids closer to the amino terminus (20 or less) (38–40). Because of nonspecific binding of the second antibody of the SPOTs kit, results of cross-reactivity in the carboxyl region of osteocalcin were inconclusive; however, there was evidence of a carboxyl-terminal epitope, shown by the behavior of peptide 36–49 in the RIA. This result is similar to that of Gundberg and Weinstein (32) and other carboxyl-terminal assays that have been reported (30, 41).

The antibody described in this report appears to be somewhat conformation specific, based on results of the effect of EDTA, in which there was a decrease in maximum binding; however, there were no differences in binding curves explaining why EDTA plasma values were not different from those in serum. The fact that the carboxylated and decarboxylated curves were identical and yet the assay was calcium dependent was explained by Delmas et al. (42), who theorized that the structure of decarboxylated osteocalcin is similar to the structure resulting from binding of calcium to fully carboxylated osteocalcin. Many assays are calcium dependent (25, 31, 43–48), and another RIA (41) has a carboxyl-terminal epitope due to antibodies raised against the synthetic peptide 37–49, and is sensitive to EDTA, which is interpreted as the conformational changes in the amino terminus being reflected in the carboxyl terminus.

The osteocalcin assay was further characterized by examining the influence of different anticoagulants used in blood collection. There were no differences between osteocalcin concentrations in serum or in EDTA plasma, in contrast to two other reports of enzyme immunoassays in which concentrations in EDTA plasma were either lower (49) or not measurable (45). In this assay, osteocalcin concentrations in heparinized plasma were slightly (8%) but significantly higher that those in serum. Other reported results have been mixed—either higher (50), lower (46, 49), or equal (30, 47, 51, 52) to serum concentrations, although all differences were <15%. Use of sodium citrate as an anticoagulant had no effect in this assay, although decreases of up to 30% compared with serum have been reported (47, 49). Similarly, Thiede et al. (53) have reported this trend with citrate in humans as well as in rats, where differences were even larger in females. They proposed that the higher concentrations in serum may be due to the release of osteocalcin from platelets during activation. Tracy et al. (31) and Power et al. (49) suggest that the difference between serum and plasma may be due to erythrocyte hydrolases released when anticoagulants are present. Differences in anticoagulant effects seen between assays may reflect different assay properties such as calcium dependence of the assay, degree of hemolysis, or differences in processing or storage conditions. Differences due to gender, as observed in rats (53), were not seen in the subjects studied here.

Methods for measuring the carboxylation state of osteocalcin have focused on the differential binding of osteocalcin and undercarboxylated osteocalcin to hydroxyapatite (8, 54), although complete separation of the two forms is difficult because of incomplete binding of carboxylated forms at lower concentrations and nonspecific binding of undercarboxylated osteocalcin at higher concentrations. Hydroxyapatite may also have other binding sites for osteocalcin irrespective of the gla domain. In addition, the behavior of partially carboxylated species is unknown. Direct assays have not been reported, with the exception of one account in abstract form of a two-site ELISA with one monoclonal antibody directed against the amino acids 14–30 (55).

Price et al. first described the hydroxyapatite binding technique (54) and its utility in detecting changes in carboxylation of osteocalcin in rats on warfarin. Merle and Delmas (8) found the best discrimination by using an average of the results from two concentrations of hydroxyapatite with <10% of carboxylated osteocalcin not binding to hydroxyapatite and 50% of undercarboxylated osteocalcin binding to the hydroxyapatite. The use of barium sulfate as a binder was investigated here with similar findings for carboxylated osteocalcin; however, a greater amount of undercarboxylated osteocalcin remains in the supernatant. Although the hydroxyapatite procedure used in our laboratory was slightly different from that of Merle and Delmas (8), concentrations of undercarboxylated osteocalcin were lower than those measured with the barium sulfate procedure. The lower concentrations appear to support their suggestion that the hydroxyapatite procedure underestimates undercarboxylated osteocalcin (9). As with hydroxyapatite, the use of barium sulfate to isolate undercarboxylated osteocalcin does not reflect the true degree of undercarboxylation of the protein. However, relative measures are reflective of changes in carboxylation state, as discussed below.

Knapen et al. (56), however, have challenged the assumption that the material remaining in the supernate after hydroxyapatite adsorption ("free" portion) is partly or totally undercarboxylated osteocalcin, and propose that it is a form of osteocalcin that has lost its gla domain by proteolysis. This was hypothesized in a follow-up to their original study (3), which showed an increase in hydroxyapatite binding capacity in postmenopausal women supplemented with 1 mg of vitamin K1 for 2 weeks. They concluded that vitamin K affected only the bound portion. In contrast to this report, we observed that the species measured in the supernate, presumed to be undercarboxylated osteocal-
cin, is highly responsive to changes in vitamin K status. The undercarboxylated portion was demonstrated to increase with warfarin dosing and decrease with vitamin K₄ administration. However, total osteocalcin concentrations did not change. Therefore, undercarboxylated osteocalcin appears to be a sensitive marker for vitamin K status. Despite the report by Knapen et al. questioning the insensitivity of the free osteocalcin portion (56), an improvement in vitamin K status, measured by an increase in hydroxyapatite binding capacity, was observed in postmenopausal women (3) and in pregnant women and their newborn babies (7). No change was observed in premenopausal women, however (3). Supplementation of cystic fibrosis patients with 5 mg of vitamin K₄ per week for 4 weeks also significantly reduced the percentage of undercarboxylated osteocalcin in comparison with a 4-week unsupplemented period (11). Similarly, supplementation of vitamin K₄ with food, in the form of broccoli or fortified corn oil, to concentrations of 5 times the recommended daily allowance for 5 days also resulted in a significant decrease in undercarboxylated osteocalcin concentrations in young subjects (12).

Warfarin acts as an inhibitor of the vitamin K cycle; thus proteins are produced lacking some or all gla residues. Studies on the alteration in vitamin K status through antagonism with oral anticoagulants have consistently shown undercarboxylated osteocalcin to reflect this antagonism (4–9), and this study, even with the minidose regimen, was no exception. In two short-term, longitudinal studies, one in which subjects received a 30-mg dose of warfarin and blood samples were taken 72 h after dosing (4) and one in which doses and days of treatment were not defined (6), undercarboxylated osteocalcin concentrations increased. Similarly, long-term studies of patients on stable oral anticoagulant therapy have also shown undercarboxylated osteocalcin concentrations to be increased in comparison with controls (4–9). Only two of these studies defined the doses of anticoagulants given, which in general were >1 mg/day, and, as they did not specify in the others, it was assumed that the patients were not uniformly on low-dose regimens as in this study. In addition to changes in undercarboxylated osteocalcin, the total amount of osteocalcin decreased in some (4, 6, 7), but not all (4, 7, 8), studies. Previously, warfarin was shown to affect the secretion of osteocalcin from the osteoblast in an in vitro study (57). Gender and age differences between the treatment and control groups may explain some of the similar concentrations (4, 8, 9). In this study total osteocalcin concentrations did not change, possibly reflecting the decreased dose or short time of administration.

Undercarboxylated osteocalcin, as measured by RIA after the differential binding of carboxylated and undercarboxylated forms to barium sulfate, shows promise as a sensitive measure of vitamin K nutritional status, as evidenced by the response to antagonism and repletion of vitamin K. In this study the percentage of undercarboxylated osteocalcin was shown to increase by an average of 170% compared with baseline in nine subjects after receiving 1 mg/day of warfarin for 7 days, whereas prothrombin times failed to show any changes during this period. As illustrated here, new approaches for monitoring the efficacy of low-dose regimens of anticoagulant therapy are necessary, since these dosages have little effect on currently used clotting assays (22). Thus, undercarboxylated osteocalcin may also be used in the future as a potential monitor of minidose warfarin therapy when the relation between carboxylation state of osteocalcin and thrombosis is more clearly elucidated.

Supported by the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University (Contract No. 53–3X06–1–10). M.E.C. was supported by a grant from the Gulbenkian Foundation, Lisbon, Portugal.

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CLINICAL CHEMISTRY, Vol. 41, No. 8, 1995 1127