Evidence that serum NTx (collagen-type I N-telopeptides) can act as an immunochemical marker of bone resorption

J. Daniel Clemens,1* Michael V. Herrick,1 Frederick R. Singer,2 and David R. Eyre3

Previous studies have shown that immunoassay of urinary NTx (cross-linked N-telopeptides of type I collagen) provides a responsive index of human bone resorption. Here we report by a sensitive immunoassay that NTx is present in serum and is suppressed appropriately in patients with Paget disease of bone by bisphosphonate antiresorptive therapy. The monoclonal antibody (1H11) developed against urinary NTx was applied in a sensitive chemiluminescence format. Results for human serum and urine showed parallel inhibition curves. The NTx concentrations in paired serum and urine samples from individual patients correlated well when urinary concentrations were normalized to creatinine concentrations (in premenopausal and postmenopausal women and Paget disease patients, \( r = 0.90, \ n = 60 \)). The percentage of NTx suppression from baseline values for Paget disease patients on bisphosphonate therapy was similar for serum and urine. Blood samples drawn from bone marrow at the site of Pagetic lesions in three patients with active disease had as much as 10-fold higher concentrations of NTx than did peripheral blood samples drawn at the same time. The latter finding is consistent with other evidence showing that immunoreactive NTx originates directly during the proteolytic cleavage of bone collagen by osteoclasts rather than, e.g., by degradative processes occurring later in the liver and kidney.

Imunoassays for biochemical markers of bone resorption are emerging that appear to be sufficiently specific and convenient for clinical use [1–6]. There is a need for such tests because, as the impact of osteoporosis on the aging population increases, the choice of therapies widens and better tools are sought to aid in risk prediction and prevention of osteoporosis. Recently, several new molecular markers and convenient immunoassays have been introduced, some of which may have the specificity to detect the relatively subtle changes in bone turnover rate that occur, e.g., in postmenopausal osteoporosis.

Collagen metabolites in urine are thus far the best resorption markers [7–10]. The focus has narrowed to collagen cross-linking amino acids and peptides that contain them. Pyridinolines (hydroxylsyl pyridinoline and lysyl pyridinoline) in total or as the free fraction in urine have been measured by HPLC or immunoassay [11–13]. Type I collagen telopeptide sequences that contain these cross-linking residues are also proving to be reliable and, as shown in comparative studies, are more responsive and osteoclast-specific markers of bone resorption than are the free cross-links themselves [14–20].

A resorption assay that measures cross-linked N-telopeptides of type I collagen (NTx)4 in urine was described [21]. A commercial ELISA version is available (Osteomark®, Ostex International) that can measure the excretion of NTx in spot (untimed) urines or 24-h collections. Results are normalized to creatinine concentration. A reliable immunoassay to measure NTx in serum could avoid the added variability of having to normalize urine values to creatinine and might aid in understanding the metabolism and kidney clearance of the NTx peptides. Therefore, we set out to determine whether the same analyte could be measured in serum with a sensitive research-grade chemiluminescence immunoassay. We demonstrate here the ability to measure immunoreactive NTx in human serum and present findings to show that this analyte responds appropriately in monitoring the clinical response to antiresorptive therapies in Paget disease patients.

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4 Nonstandard abbreviations: NTx, cross-linked N-telopeptides of type I collagen; HRP, horseradish peroxidase; BCE, bone collagen equivalents.
Materials and Methods

Clinical Samples
Patients were selected with mild to severe Paget disease. Severely affected patients were identified on the basis of having particularly high serum total alkaline phosphatase activities (>1000 U/L). All serum, marrow blood, and urine specimens were collected under investigational review board approval from the clinical sites. Specimens were stored frozen (−20 °C) before analysis.

Serum Immunoassay Procedure
A chemiluminescence immunoassay in competitive inhibition format was developed to use the mouse monoclonal antibody, mAb 1H11, and the NTx antigen prepared from human bone by bacterial collagenase digestion, essentially as described previously [21]. MAb 1H11 was purified and conjugated to horseradish peroxidase (HRP) by a modification of the method described by Nakane and Kawaoi [22]. Sterile polystyrene tubes (12 × 75 mm) were coated with the NTx antigen by passive adsorption at 0.5 nmol BCE/L in 100 mmol/L phosphate buffer, pH 8.0. For assay use, the labeled antibody, mAb 1H11–HRP, was diluted to 5 μg/L in 2 mmol/L phosphate, 2.5 mL/L Tween-20, 10 mmol/L 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate buffer. Test specimens and assay calibrators were diluted 1:22.5 in 2 mmol/L phosphate buffer, pH 8.0, and then combined in an equivalent (1:1) ratio with diluted mAb 1H11–HRP in coated tubes. Dilution was required to minimize an interference by serum protein in the assay. At all dilutions of test specimens and calibrators, assays were run in duplicate. Mean values were used for the final results. The samples were incubated for 1 h in a refrigerator at 4 °C to allow competition for antibody binding sites. The contents of each tube were then aspirated and the tubes were washed twice with deionized water. Luminescence development buffer—0.06 mmol/L luminol (Sigma Chemical Co.), 0.12 mmol/L iodo phenol (Aldrich Chemical Co.) and 0.6 g/L hydrogen peroxide in 100 mmol/L phosphate, pH 8.0—was then added to each tube. The light signal was quantified immediately with a Berthold Lumat LB 9501 luminometer purchased from EG&G Wallac (Gaithersburg, MD). Sample measurements were read from the calibration curve with use of a 4-parameter logistic curve-fitting program. The calibration curve measurement range was 0–40 nmol BCE/L. Specimens giving results greater than the upper limit of the calibration curve were diluted further in the 2 mmol/L phosphate buffer described above. Final immunoassay results are reported in nanomoles of BCE per liter.

Urinary NTx Determinations
Urinary NTx was quantified with the Osteomark immunoassay kit. Results are reported normalized to urinary creatinine as measured by the Jaffe method [23] (with a reagent kit purchased from Sigma) and expressed as nmol BCE/mmol creatinine.

The Osteomark immunoassay imprecision (CV) was <8%, and the lower limit of detection was 20 nmol BCE/L. Urinary creatinine assay imprecision was <4%.

Collection of Marrow Blood Samples from Pagetic Lesions
Informed consent was obtained from three patients with Paget disease who had radiological evidence of the disease in at least one iliac crest and serum alkaline phosphatase activity at least twice the upper reference limit. Bone marrow aspiration was carried out from the posterior superior iliac crests exhibiting Paget disease under xylocaine local anesthesia. The marrow was aspirated into a syringe containing a known volume of α Minimum Essential Medium that included heparin 1000 IU/mL and fetal calf serum 50 mL/L. After centrifugation, the plasma was stored frozen (−70 °C) until analysis.

Results

Assay Performance
The reproducibility of the chemiluminescence immunoassay is presented in Table 1. The mean intraassay and interassay CVs were 7.7% and 13.4%, respectively. The lower limit of detection of the method was 0.84 nmol BCE/L (range 0.05–2.1 determined over 10 assays). Antigen recovery was 87% (range 77–94%) in four serum specimens and 98% (range 89–121%) in four plasma specimens. Mean dilutional recoveries for a Paget disease serum sample were 113%, 99%, and 108% at 1:2, 1:4, and 1:8 dilutions, respectively. Parallel inhibition curves for serum and urine (not shown) and acceptable recoveries on addition to serum specimens of the antigen used to calibrate the urine assay

[^2]: BCE: Bone Collagen Equivalents, units of immunoreactive NTx calibrated in moles of type I collagen in human bone that on digestion in vitro generate the same assay response as immunoreactive NTxs [21].
(Osteomark) demonstrate that mAb 1H11 recognizes essentially the same epitope in serum and urine. Moreover, fractionation of NTx species from Paget disease serum by molecular sieve and reversed-phase chromatography indicated immunoreactivity in the same low-molecular-mass range with chromatographic properties similar to those of the NTx peptides from urine (data not shown).

This is consistent with the conclusion that the epitope recognized by mAb 1H11 requires proteolysis of the cross-linked α2(I) N-telopeptide domain of type I collagen to an 8 amino acid sequence [21, 29].

COMPARATIVE RESULTS FOR PREMENOPAUSAL, POSTMENOPAUSAL, AND PAGET DISEASE PATIENTS

To test whether the serum NTx marker was clinically discriminatory, we assayed matched collections of urine and serum from different Paget patients who varied in disease severity (according to their degree of increase of bone turnover markers). Postmenopausal subjects with increased bone resorption, as judged by their urinary NTx values, were compared with a group of premenopausal women who exhibited normal rates of bone resorption. Serum and urine NTx results were compared from 32 premenopausal women, 21 postmenopausal women, and 7 patients with Paget disease of bone. Table 2 compares the mean values for serum and urine for these subject groups. Mean values were higher in the Paget disease patients than in the premenopausal group (4.5-fold greater in serum, 5.9-fold greater in urine), and also in the postmenopausal group compared with the premenopausal group (1.6-fold greater in serum, 2.0-fold greater in urine). The correlation between paired serum and urine samples for the entire population (n = 60) was r = 0.90 (Fig. 1).

The response to antiresorptive therapy was assessed in three additional patients with Paget disease of bone, who were monitored during an 8-week course of oral resorionate (30 mg daily) and for 4 months afterwards. Suppression in bone resorption was seen in both serum and urinary NTx values (Fig 2). The paired serum and urine

Table 1. Reproducibility of the chemiluminescence immunoassay for NTx in human serum.

<table>
<thead>
<tr>
<th>Serum sample</th>
<th>NTx conc., nmol BCE/L</th>
<th>CV, %</th>
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<tbody>
<tr>
<td></td>
<td>Intraassay</td>
<td>Interassay</td>
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<tr>
<td>1</td>
<td>4.7</td>
<td>9.2</td>
</tr>
<tr>
<td>2</td>
<td>5.6</td>
<td>15.9</td>
</tr>
<tr>
<td>3</td>
<td>7.7</td>
<td>6.5</td>
</tr>
<tr>
<td>4</td>
<td>8.5</td>
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<td>5</td>
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<td>6</td>
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<td>7</td>
<td>21.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Mean</td>
<td>7.7</td>
<td>13.4</td>
</tr>
</tbody>
</table>

Table 2. Comparison of NTx assay results for paired serum and urine samples from different subject groups (mean ± SD).

<table>
<thead>
<tr>
<th>Subject group</th>
<th>n</th>
<th>Serum, nmol BCE/L</th>
<th>Urine, nmol BCE/mmol creatinine</th>
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</thead>
<tbody>
<tr>
<td>Premenopausal women</td>
<td>32</td>
<td>4.7 ± 2.2</td>
<td>36.2 ± 17.4</td>
</tr>
<tr>
<td>Postmenopausal women</td>
<td>21</td>
<td>7.3 ± 2.8</td>
<td>72.0 ± 28.2</td>
</tr>
<tr>
<td>Paget disease of bone</td>
<td>7</td>
<td>21.5 ± 15.8</td>
<td>214.0 ± 90.2</td>
</tr>
</tbody>
</table>

Fig. 1. Correlation of serum and urine NTx measurements on paired samples from individual subjects.

The subject group (n = 60) included premenopausal (n = 32) and postmenopausal (n = 21) women and Paget disease patients (n = 7).
values for each patient were highly correlated ($r = 0.90, 0.94, \text{and} 0.97$).

**Table 3. Comparative NTx values for marrow blood vs peripheral blood from three Paget disease patients.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>NTx, nmol BCE/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Marrow blood</td>
</tr>
<tr>
<td>A</td>
<td>645</td>
</tr>
<tr>
<td>B</td>
<td>360</td>
</tr>
<tr>
<td>C</td>
<td>184</td>
</tr>
</tbody>
</table>

In patient A, only the left pelvis was affected. In patients B and C, multiple bones were affected in addition to the pelvic site of marrow blood sampling.

**Discussion**

The results show that the immunoreactive peptide analyte, NTx, originally identified in urine as a product of osteoclastic bone resorption, can also be measured in human serum. A sensitive immunoassay can quantify the concentrations in serum reliably. Premenopausal, postmenopausal, and Paget disease patients can be distinguished well by serum analysis and by urine analysis, although the data presented here show a somewhat greater discriminatory power for urine NTx than for serum NTx. A growing body of clinical studies has shown that urinary NTx provides a sensitive marker of human bone resorption [4, 6, 14–19, 24–28]. Its specificity is based on originating solely from type I collagen, being particularly enriched as a cross-linking structure in bone type I collagen [29] and being produced directly as a proteolytic neoepitope by osteoclast activity during bone resorption [30]. Analysis of urine specimens has certain advantages, including ease of collection and reduced biohazard precautions for clinical laboratory personnel than with blood-based specimens. However, a serum marker with similar specificity would have other advantages, e.g., avoiding the potential added variability with urine from having to normalize results to creatinine or in collecting 24-h specimens, and greater availability of blood specimens, which are often drawn for other measurements anyway. Finally, in renal failure patients, who are at risk of metabolic bone disease, urine may not be an option and serum could be informative.

The finding of a high NTx concentration in marrow blood taken from a bony lesion site in Paget disease, compared with peripheral blood, supports the concept that NTx is a proteolytic neoepitope generated by osteoclasts as they resorb bone. A study of mouse osteoclasts cultured on human bone or dentin also showed the release of NTx into the culture medium [30]. The yield of immunoreactive NTx was also quantitative, as judged by the amount of resorbed collagen in the medium and its theoretical NTx content. This latter study also showed that the cultured osteoclasts degraded the resorbed bone collagen to peptide-bound pyridinolines but not to free pyridinolines.

The serum concentration range of NTx compared with 24-h urine values for normal subjects indicates a plasma clearance exceeding 28 mL/min, assuming zero protein binding and no renal metabolism. This implies a rapid
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References


