Tartrate-resistant Acid Phosphatase Isoform 5b as Serum Marker for Osteoclastic Activity

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Background: Tartrate-resistant acid phosphatase (AcP) 5b is a marker of osteoclastic activity and bone resorption. Immunoassays for serum TRAcP may lack sensitivity and specificity because of the presence of non-bone isoform 5a. The purpose of this study was to isolate the serum isoforms, quantify their disease-related expressions, and test an improved immunoassay for TRAcP 5b.

Methods: We separated TRAcP isoforms chromatographically from pooled sera of healthy, rheumatoid arthritis (RA) and endstage renal disease (ESRD) subjects. TRAcP isoforms were identified by electrophoresis and quantified by biochemical and immunochemical assays. Serum TRAcP activity in healthy, RA, and ESRD cohorts was assessed at pH 5.5 and 6.1, and compared with bone alkaline phosphatase (BAP) and N-telopeptides of type I collagen (NTx).

Results: TRAcP isoforms 5a and 5b were present in all sera; 5b was identical to osteoclastic TRAcP. In serum from healthy subjects, 5a accounted for 87% of the enzyme protein but only 55% of the activity. In RA, both isoforms were increased two- to threefold in protein, but their specific activities were subnormal. In ESRD, only 5b was abnormal, being increased fivefold in protein and threefold in activity. In RA sera, TRAcP activity did not correlate with either BAP or NTx. In ESRD sera, TRAcP activity correlated with BAP and NTx only when measured at pH 6.1.

Conclusions: All sera contained both TRAcP isoforms 5a and 5b, but only 5b was present in bone. TRAcP isoform expression was variable in different diseases. Measurement of TRAcP activity at pH 6.1 improves the specificity of immunoassay for isoform 5b.

Osteoporosis and its complications affect 25 million people and account for $10 billion in health expenditures annually in the United States alone (1). In addition to postmenopausal women, people at risk to develop osteoporosis include those with chronic renal failure, rheumatic diseases, age-related hypogonadism, and malignancy with bone metastasis (2–5). In such cases, there is a need for serum markers of accelerated bone turnover to assess fracture risk and monitor treatment. Tartrate-resistant acid phosphatase (TRAcP)4 has been advocated as a biomarker of osteoclastic activity with clinical value for assessment of bone resorption (6–8). Immunoassays have been developed for serum TRAcP, but are not yet used clinically because they lack sensitivity and specificity (9–16). Serum TRAcP is composed of two antigenically related isoforms: 5a and 5b (17). Our early experience and that of others indicate that serum isoform 5b is derived from osteoclasts, whereas the origin of 5a is unknown (18, 19). The clinical sensitivity of TRAcP immunoassays could be increased if they were made to measure specifically TRAcP 5b of bone origin (19). In addition, the proportional expression of TRAcP 5a and 5b may not be the same in all diseases, thus increasing the difficulty in interpreting results. To use TRAcP effectively as a serum marker for osteoclastic activity, we need to separate and study the individual isoforms to improve the specificity and sensitivity of immunoassays for bone TRAcP.

Here we further document the clinical value of an improved immunoassay method for selectively measuring TRAcP 5b activity and introduce the concept that TRAcP 5a may have its own clinical significance. We did this by separating TRAcP isoforms 5a and 5b from pooled sera of healthy subjects with column chromatography and studying their properties independently. We compared the electrophoretic properties of serum isoforms to that of normal osteoclastic TRAcP and evaluated the quantitative changes and the clinical significance of TRAcP 5a and 5b.
in serum from patients with rheumatoid arthritis (RA) and end-stage renal disease (ESRD).

Materials and Methods

Collection of Serum

Two batches of pooled normal serum (500 mL and 300 mL) collected from >350 donors were prepared from waste specimens obtained from the Louisville-area Red Cross. All sera were held at 4 °C for no more than 48 h before their release. Two batches of pooled serum (500 mL each), consisting of >250 randomly acquired waste specimens from patients with assorted rheumatic diseases, were also prepared. A single RA serum pool (250 mL) was prepared from >60 randomly selected specimens acquired in the same way as the rheumatic disease sera. A single serum pool (150 mL) of >150 specimens from patients undergoing hemodialysis for ESRD was prepared from sera collected immediately before or immediately after the dialysis procedure.

Individual serum specimens were collected from 27 healthy donors, 35 patients with RA, and 30 patients with ESRD obtained before hemodialysis. Before the blood samples were taken, informed consent was obtained from each healthy donor and from each ESRD patient. RA sera were waste specimens obtained after they had served their clinical purposes. All sera were stored at −70 °C within 8 h of collection. These studies were approved by the Human Studies Subcommittee of the VA Medical Center, Louisville.

TRAcP Assays

Total TRAcP activity was determined using 4-nitrophenyl phosphate (4-NPP) as substrate according to the microplate assay method of Lau et al. (20) with modifications. Sample (50 μL) was incubated for 1 h at 37 °C with 150 μL of substrate consisting of 7.6 mmol/L 4-NPP in 100 mmol/L sodium acetate buffer containing 50 mmol/L sodium tartrate and adjusted to pH 4.9, 5.5, or 6.1. The reaction was stopped by the addition of 50 μL of 3 mol/L NaOH. A calibration curve was constructed from solutions of 4-nitrophenol containing 10–0.16 μmol/min per liter of sample (U). Absorbance was measured at 405 nm in a Bio-Rad Model 550 Microplate Reader.

Immunoaassays for type-5 TRAcP activity and protein were performed simultaneously according to previously published methods (16). A common anti-TRAcP monoclonal antibody (14G6) was used to coat duplicate micro-wells. Samples were incubated in coated wells overnight at 4 °C. After washing, TRAcP activity was measured by adding the 4-NPP substrate at pH 5.5 or 6.1. TRAcP protein was determined with a second, peroxidase-conjugated anti-TRAcP antibody, J1B (provided by Dr. J. Halleen, University of Turku, Finland).

Chromatographic Separation and Identification of TRAcP 5a and 5b

TRAcP isoforms 5a and 5b were separated from each serum pool by published methods of cation-exchange chromatography (17) with modifications. Serum was acidified to pH 5.0 by dropwise addition of glacial acetic acid. Precipitated proteins were removed by centrifugation at 10 000g for 15 min. The supernatant was applied to a column (1.6 × 10 cm) of Sepharose S fast flow (Amersham Pharmacia) equilibrated with 10 mmol/L sodium acetate, pH 5.0. The column was washed with 200 mL of equilibration buffer followed by 200 mL of equilibration buffer containing 0.3 mol/L NaCl to remove weakly bound proteins. TRAcP isoforms were then eluted by a linear NaCl gradient from 0.3 to 0.75 mol/L NaCl; 100 equal fractions were collected in a total volume approximately equal to the serum sample to facilitate direct comparisons of TRAcP isoform yield. The identities of eluted TRAcP enzyme peaks, as well as purified bone TRAcP (a generous gift from Dr. J. Halleen, University of Turku, Finland), were confirmed by nondenaturing polyacrylamide gel electrophoresis (21). A 5-μL aliquot from the fraction at the apex of each enzyme peak was mixed with 45 μL of 100 g/L bovine albumin and 50 μL of sample gel. Electrophoresis was carried out at pH 4.0 for 90 min to enhance the separation of TRAcP isoforms. Gels were immediately stained for TRAcP activity in 100 mmol/L sodium acetate-50 mmol/L sodium tartrate (pH 5.5) containing 2 g/L naphthol ASBⅠ-P and 0.02 g/L Fast Garnet GBC.

Biochemical Characterization of TRAcP Isoforms 5a and 5b

Column fractions of each enzyme peak were pooled, and the volumes were recorded. The pH optimum of each TRAcP isoform was determined at pH 4.0–6.5, using the total activity assay. The yield of enzymatic activity in each peak was calculated from peak volume and results of total activity assay at its pH optimum. The yield of enzyme protein in each peak was calculated from the peak volume and results of a two-site immunoassay. Yields were normalized to a 1-L sample. The specific activity of each TRAcP isoform at its pH optimum was calculated as the ratio of activity/protein and expressed as U/μg. The Km for each partially pure enzyme was determined from Lineweaver-Burke plots after assay of a single sample at its optimum pH at substrate concentrations of 0.2–10 mmol/L 4-NPP.

Other Bone Markers

Serum bone alkaline phosphatase (BAP) activity was determined in individual sera as a measure of bone formation (22) by a method (Metra Biosystems) based on the enzyme-capture immunoassay principle. N-telopeptides of type-I collagen (NTx) were measured in individual sera as a measure of bone resorption (23) by a method (Ostex International) based on a competitive antigen-binding principle.

Results

Evaluation of TRAcP Isoforms in Serum from Healthy Subjects

Serum from healthy subjects was separated chromatographically twice with similar results. Typical findings,
achieved. Assays to 6.1, preferential detection of osteoclastic 5b was twofold. Therefore, by increasing the pH of TRAcP activity two-fold, whereas TRAcP 5b activity was increased two-fold. At pH 6.1, TRAcP 5a activity was reduced.

ISOFORMS Differed Dramatically (Fig. 3). At pH 4.9, TRAcP 5a and TRAcP 5b contributed almost equally to total activity. At pH 6.1, TRAcP 5a activity was reduced twofold, whereas TRAcP 5b activity was increased two-fold. Therefore, by increasing the pH of TRAcP activity assays to 6.1, preferential detection of osteoclastic 5b was achieved.

EVALUATION OF TRAcP ISOFORMS IN PATHOLOGIC CONDITIONS

TRAcP 5a and 5b were separated chromatographically twice using pooled rheumatic disease serum and once using selected RA serum with similar results. Fig. 4 illustrates the results from RA serum, in which TRAcP 5a accounted for 90% of the TRAcP protein and 63% of TRAcP activity. Both TRAcP isoforms were increased in absolute amounts over the concentrations seen in the control serum (Table 1). The specific activities of both isoforms 5a and 5b in serum from patients with rheumatic diseases and RA were approximately one-half the activities in the serum from healthy subjects. The electrophoretic mobilities of isoforms 5a and 5b in rheumatic diseases and RA were the same as for the isoforms from healthy subjects. The $K_m$ of isoform 5a was increased to 3.2 mmol/L 4-NPP, whereas that of isoform 5b was unchanged. Therefore, the significant increase in TRAcP protein we observed previously in rheumatic diseases (16) and RA (24) was attributable to an increase in the amount of both isoforms 5a and 5b with a concomitant decrease in the specific activities of both.

Compared with pooled control serum, chromatographic separation of TRAcP 5a and 5b from pooled ESRD serum showed marked relative and absolute increases in 5b protein and activity (Table 1 and Fig. 5). In this condition, TRAcP 5a accounted for 60% of total TRAcP protein and 27% of activity. In absolute terms, isoform 5a protein was unchanged, whereas isoform 5b protein was increased almost fivefold compared with control serum. In contrast to RA serum, the specific activity of isoform 5a in ESRD serum was similar to that of the control serum (0.47 vs 0.58 U/$\mu$g of TRAcP, respectively), whereas the specific activity of isoform 5b was reduced by 42% (1.90 vs 3.30 U/$\mu$g of TRAcP, respectively). The electrophoretic properties of the TRAcP isoforms in ESRD were similar to the control serum. The $K_m$ of isoform 5a was slightly increased to 3.7 mmol/L 4-NPP, whereas that of isoform 5b decreased slightly to 4.4 mmol/L 4-NPP. Therefore, the increased TRAcP activity we had observed previously in serum from ESRD patients (16, 25) was attributable to an absolute increase of circulating 5b isoform. Its reduced

Table 1. Abundance of TRAcP isoforms in pooled serum from healthy control subjects and from RA and ESRD patients.

<table>
<thead>
<tr>
<th></th>
<th>5a</th>
<th>5b</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.91</td>
<td>1.56</td>
</tr>
<tr>
<td>RA</td>
<td>1.30</td>
<td>4.71</td>
</tr>
<tr>
<td>ESRD</td>
<td>0.78</td>
<td>1.64</td>
</tr>
</tbody>
</table>

**Table 1. Abundance of TRAcP isoforms in pooled serum from healthy control subjects and from RA and ESRD patients.**

- **5a** and **5b** activities were determined by immunoassay as described in Materials and Methods.

Fig. 1. Chromatographic profile of type-5 TRAcP in a pooled control serum.

Cation-exchange chromatography of a 300-mL serum sample from healthy controls with a 300-mL NaCl gradient resolved two TRAcP activity peaks. The first broad peak contained 87% of the total TRAcP protein but only 55% of the activity. The second peak contained 13% of the protein but 45% of TRAcP activity. Note the significant difference in the specific activities of the enzyme peaks, TRAcP activity and protein in column fractions were determined by immunoassay as described in Materials and Methods.

Fig. 2. Nondenaturing PAGE gels identifying peak 1 (fraction 30) and peak 2 (fraction 64) from chromatogram of control serum as TRAcP 5a and 5b, respectively.

Pure bone TRAcP is identical to isoform 5b. Electrophoresis was carried out and gels stained as described in Materials and Methods.
specific activity suggests that serum from ESRD patients may contain substantial amounts of inactive isoform 5b.

**SIGNIFICANCE OF DIFFERENTIAL EXPRESSION OF TRAcP ISOFORMS IN PATHOLIC CONDITIONS**

In both RA and ESRD, an adjustment to pH 6.1 for determination of total enzymatic activity selectively increased the proportion of isoform 5b detected (Fig. 6). Peak activity of TRAcP 5b increased twofold, and peak activity of TRAcP 5a decreased twofold. Measuring TRAcP activity in the enzyme capture immunoassay at pH 6.1, therefore, provided greater specificity for isoform 5b, as shown previously in postmenopausal women (19).

To determine whether this adjustment produced a stronger association between TRAcP activity and bone turnover in other diseases, we performed immunoassays for total type-5 TRAcP activity at pH 5.5, isoform 5b activity at pH 6.1, and TRAcP protein in a group of 27 control subjects, 35 RA patients, and 30 ESRD patients on hemodialysis. Serum BAP and NTx were also determined as independent markers of bone formation and resorption (Table 2). Mean total TRAcP activity (pH 5.5) was not increased in either RA or ESRD; however, a subpopulation of ESRD patients who had increased total TRAcP caused the wide distribution and larger standard deviation. On the other hand, mean TRAcP 5b activity (pH 6.1) and TRAcP protein were significantly increased in both cohorts. BAP was increased in both RA and ESRD, whereas NTx was increased only in ESRD. If TRAcP activity measured at pH 6.1 is selective for osteoclastic isoform 5b and related to bone turnover, it should correlate with serum BAP or NTx. The calculated correlation coefficients for the tested markers are summarized in Table 3. Whereas no significant correlations were found between TRAcP and bone markers in RA, both total TRAcP and TRAcP 5b activities correlated significantly with bone markers in ESRD. No significant correlations were obtained between TRAcP protein and bone markers in any disease.

**Discussion**

In previous studies of TRAcP as a serum monitor for osteoclastic activity, we developed two immunoassays, one to measure type-5 TRAcP activity and the other to measure type-5 TRAcP protein (16). We used these assays to quantify serum TRAcP in patients with ESRD and those with rheumatic diseases, including RA. Twenty-six percent of ESRD patients had a significant increase of TRAcP activity in those with rheumatic diseases, including RA. Twenty-six percent of ESRD patients had a significant increase of TRAcP activity, whereas 31% of patients with rheumatic diseases had increased TRAcP protein without increased TRAcP activity. Because circulating type-5 TRAcP exists as two isoforms, 5a and 5b, the differences observed in TRAcP activity and protein in these diseases may be attributable...
to changes in the proportional expression of the two isoforms. To analyze their properties independently, we isolated isoforms 5a and 5b from serum from healthy controls and serum from two groups of patients at risk of increased bone turnover. Using ion-exchange chromatography, nondenaturing electrophoresis, and immunoassays, we were able to conclude the following: (a) serum isoform 5b is identical to bone TRAcP; (b) the basis for increased TRAcP in these two groups of patients is attributable to expression of different isoforms; and (c) an improved method to estimate osteoclastic TRAcP 5b activity at pH 6.1 (19) is further substantiated.

Our present results confirm earlier findings (18) showing that TRAcP 5a and 5b are antigenically related; both react with the antibodies used for immunoassays. We demonstrated that they have different specific activities and that their absolute and proportional amounts differed in disease-specific ways. We had previously reported that the specific activity of TRAcP in serum samples from healthy subjects and from patients was ~10-fold lower than estimated here. We have since found that the affinity-purified TRAcP 5b used to calibrate the two-site immunoassay in our previous work contained substantial amounts of denatured TRAcP protein. Therefore, the amount of immunoreactive TRAcP protein in the calibrator was lower than the total protein quantified by ultraviolet absorbance, which led to overestimation of serum TRAcP protein by the two-site immunoassay. For this work, affinity-purified TRAcP 5b was freshly prepared from hairy cell leukemia spleen for use as the calibrator. Despite these differences between studies in the absolute amounts of measured TRAcP protein, the relationships between TRAcP specific activity and disease and the biological significance of differential isoform expressions are unchanged.

TRAcP is believed to be a biomarker of osteoclastic activity. Its activity is increased in the serum of growing children (17, 26) and in patients with various metabolic bone diseases (6–8, 27). Lam et al. (18) showed that the TRAcP of giant cell tumor was isoform 5b and that it had immunologic identity to serum TRAcP 5b. Here we confirm and extend those findings by showing that serum TRAcP 5b is the same as normal bone TRAcP, as originally defined by electrophoresis. Serum TRAcP activity often is increased in patients with ESRD undergoing hemodialysis (7, 16, 27, 28). This is presumed to result from increased bone resorption. Results from our chromatographic and electrophoretic studies are strong evidence that increased TRAcP activity in ESRD serum is in fact attributable to increased TRAcP 5b. The reduced specific activity of TRAcP 5b in ESRD serum suggests some partial inactivation of isoform 5b in this disease.

Our previous work with simultaneous assays of TRAcP activity and protein in rheumatic diseases showed

### Table 2. TRAcP, BAP, and NTx concentrations in control, RA, and ESRD sera.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control (n = 27)*</th>
<th>RA (n = 35)</th>
<th>ESRD (n = 30)</th>
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<tbody>
<tr>
<td>Total TRAcP activity (pH 5.5), U/L</td>
<td>3.13 ± 0.54</td>
<td>3.25 ± 0.93</td>
<td>3.81 ± 1.92</td>
</tr>
<tr>
<td>TRAcP 5b activity (pH 6.1), U/L</td>
<td>1.63 ± 0.52</td>
<td>2.16 ± 0.71b</td>
<td>3.50 ± 1.31c</td>
</tr>
<tr>
<td>Total TRAcP protein, µg/L</td>
<td>10.33 ± 4.21</td>
<td>28.86 ± 32.4b</td>
<td>19.57 ± 10.96d</td>
</tr>
<tr>
<td>BAP, U/L</td>
<td>21.27 ± 5.86</td>
<td>28.66 ± 10.82b</td>
<td>29.32 ± 13.88b</td>
</tr>
<tr>
<td>NTx, nmol/L BCE</td>
<td>19.33 ± 3.89</td>
<td>17.13 ± 3.86b</td>
<td>524 ± 783c</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate number of sera in cohort.

b–d Compared with control: b P < 0.001; c P < 0.0001; d P < 0.05.

e BCE, bone collagen equivalents.
a strong association between RA and increased TRAcP protein with low specific activity (24). In this study, we showed that most of this low-activity TRAcP is isoform 5a. The amount of TRAcP 5a protein was increased threefold in RA, whereas 5a activity was increased only 43%. The amount of TRAcP 5b protein in RA sera was twice that of control sera, but its specific activity was also reduced. Increased TRAcP 5b protein in RA sera may reflect RA-associated osteoporosis or bone erosion by osteoclasts (29); however, its reduced activity remains to be explained. RA is an autoimmune disease with chronic inflammation. Activated macrophages are abundant in rheumatoid synovial tissues (30) and are known to contain TRAcP (31). The origin of serum TRAcP 5a is unknown. It coexists with, but is distinct from TRAcP 5b. The abnormal macrophages of Gaucher disease accumulate abundant TRAcP, which has been characterized as isoform 5a (32). It is conceivable that the increased amounts of both TRAcP 5a and 5b in the circulation of RA patients could be derived from activated macrophages rather than, or in addition to, osteoclasts.

The fact that TRAcP 5b often contributes much toward total activity but little toward total protein explains why TRAcP activity assays correlate better with other markers of bone turnover than do TRAcP protein assays. TRAcP 5b has a higher pH optimum than TRAcP 5a (17). Recently, this difference has been exploited to increase the specificity of a TRAcP immunoassay for isoform 5b (19). In that study, a significant decrease in TRAcP 5b activity (pH 6.1) was observed in response to hormone replacement therapy among postmenopausal women. Total TRAcP activity (pH 5.5) did not change significantly with hormone replacement therapy. It was concluded that measurement of TRAcP 5b activity at pH 6.1 more closely reflected bone resorption (19).

In the present study, we confirmed that immunoassay of TRAcP activity at pH 6.1 significantly increases the specificity for isoform 5b. We then examined the associations among total TRAcP activity at pH 5.5, TRAcP 5b activity at pH 6.1, and total TRAcP protein and other bone metabolic markers in RA and ESRD sera. Although TRAcP 5b activity was significantly increased in both RA and ESRD, it may have quite different clinical and biological significance in these diseases. In RA sera, TRAcP 5b activity did not correlate with BAP or NTx. This could be explained by the fact that most TRAcP in RA sera is isoform 5a and unrelated to bone resorption. Even when measured at pH 6.1, a large amount of isoform 5a would still contribute somewhat to the measurement of isoform 5b. Alternatively, TRAcP 5b in RA could be derived from inflammatory macrophages instead of osteoclasts. Some tissue macrophages contain abundant TRAcP activity (31, 33), and TRAcP has been purified from alveolar macrophages (31, 34) as well as from osteoclasts (14, 35).

In this regard, it is noted that the mean NTx of RA sera (17.13 ± 3.86 nmol of bone collagen equivalents/L) was actually lower than that of our healthy group (19.33 ± 3.89 nmol of bone collagen equivalents/L), which suggests that our RA patients may not have ongoing increased bone resorption. In ESRD sera, TRAcP 5b activity did correlate significantly with both BAP and NTx, although the strength of the associations were weak. This could be attributable to the reduced specific activity of TRAcP 5b in ESRD. In addition, because NTx are usually cleared by the kidney, their serum concentrations in renal failure may be disproportionately high relative to actual bone resorption (36). The weak correlations notwithstanding, TRAcP 5b in ESRD by all accounts is osteoclast derived.

TRAcP is a biomarker of osteoclasts with emerging clinical importance in metabolic bone disease. However, TRAcP heterogeneity in serum has had a negative impact on the sensitivity and specificity of TRAcP as a marker of bone resorption. Although TRAcP isoform 5a retains some activity at pH 6.1, which may compromise the absolute specificity of the assay for TRAcP 5b, results from our studies provide further support for measuring TRAcP activity at pH 6.1 to increase the specificity of the TRAcP immunoassay for isoform 5b (19). Until isoform 5b-specific antibodies, substrates, or inhibitors are discovered to make TRAcP immunoassays absolutely bone specific, this strategy definitely improves the specificity of TRAcP immunoassays for diseases of increased bone resorption. It should be noted that TRAcP 5b is also produced by macrophages and could, in principle, become increased in serum of patients with inflammatory diseases such as RA. Until now, TRAcP 5a has been given little significance except as an interfering substance in TRAcP 5b measurements. Our results imply that TRAcP 5a may have practical, biological, and clinical significance of its own. Alterations in the absolute amounts and patterns of TRAcP isoform expression indicate that pathogenic mechanisms leading to increased serum TRAcP 5a or 5b are different in metabolic bone disease and chronic inflammation.
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References