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Original Article

Tartrate-Resistant Acid Phosphate Activity as Osteoclastic Marker: Sensitivity of Cytochemical Assessment and Serum Assay in Comparison with Standardized Osteoclast Histomorphometry

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Abstract. Tartrate-resistant acid phosphatase (TRAP) activity is regarded as an important cytochemical marker of osteoclasts; its concentration in serum is utilized as a biochemical marker of osteoclast function and degree of bone resorption. This study was carried out to assess the sensitivity of TRAP activity both as a cytochemical marker in histological sections and as a biochemical marker in serum in comparison with the standardized histomorphometric variables of osteoclasts. To this end we investigated 24 patients (21 women, 3 men; 60 ± 17 years of age) affected with various metabolic bone diseases. Osteoclast surface (OcS/BS) and osteoclast number (OcN/BS) were evaluated by standardized histomorphometry in iliac crest biopsies. On the basis of TRAP cytochemical activity, TRAP-positive osteoclast surface (TRAP+OcS/BS) and number (TRAP+OcN/BS) were measured. TRAP-positive cells adjacent to bone and showing one nucleus or no nuclei at all in the plane of section were included in the counts as osteoclasts. Serum TRAP activity was determined by spectrophotometric assay. Values of OcS/BS and OcN/BS were much lower than those of TRAP+OcS/BS (−50%) and TRAP+OcN/BS (−60%), respectively. Correlations between OcS/BS and TRAP+OcS/BS, and between OcN/BS and TRAP+OcN/BS, were highly significant. Serum TRAP was significantly correlated with OcS/BS, OcN/BS, and TRAP+OcN/BS. These correlations, however, were rather low. Moreover, serum TRAP did not correlate with TRAP+OcS/BS. From these results, the conclusion can be drawn that while TRAP activity is confirmed as a valid cytochemical marker for identification of osteoclasts, serum TRAP activity is an osteoclastic marker of weak sensitivity. This may be due to known factors, such as synthesis of the enzyme not being unique to osteoclasts, enzyme instability, and the presence of inhibitors in serum. Mononucleated osteoclasts do not significantly influence the serum enzyme levels.

Keywords: Bone biopsy; Histomorphometry; Osteoclasts; Serum TRAP; TRAP cytochemistry

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Introduction

Osteoclasts are heterogeneous with respect to cellular size and shape, and numbers of nuclei. In histological sections they are identified mainly on the basis of multinuclearity and cell width. Recently, this criterion has been questioned, especially because it does not allow recognition of mononucleated osteoclasts [1]. Tartrate-resistant acid phosphatase (TRAP) is detectable in large amounts in the lysosomes of osteoclasts and its activity is considered an established cytochemical marker useful for recognizing polynucleated as well as mononucleated osteoclasts in bone sections [2]. However, to our knowledge no histomorphometric studies have been carried out with the aim of comparing cell morphology with TRAP activity.

TRAP is secreted in large quantities by osteoclasts, so that its serum activity is considered a biochemical marker of osteoclast function [3]. Although this serum marker is used and accepted as an indicator of osteoclast activity, no investigation has been carried out to establish how closely it reflects bone resorption in vivo. In this respect, it must be pointed out that circulating enzyme levels can be influenced by several factors not necessarily connected with resorption. Thus, the clinical significance of TRAP activity needs to be validated by comparison with bone histomorphometry [4].

The aim of the present study was to evaluate the sensitivity of TRAP activity both as a cytochemical and biochemical marker, by comparison with standardized histomorphometric osteoclastic variables. Bone biopsies of patients with various metabolic bone diseases were used.

Materials and Methods

The study was done in 24 patients (21 women, 3 men; 60 ± 17 years of age) who were hospitalized for clinical problems related to mineral metabolism. For diagnostic purposes, all patients underwent an iliac crest biopsy, which was taken under local anaesthesia with an electric trephine (Burkardt apparatus with a needle of 4 mm inner diameter). Blood samples for assessment of biochemical markers of bone metabolism were obtained just before the biopsy.

The bone specimens were fixed in 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2. They were then dehydrated in acetone and processed for glycol–methacrylate embedding without decalcification [5]. From each biopsy, at least four sets of two serial sections, about 2 μm thick, were cut at intervals of >20 μm using a Reichert-Jung 1150/Autocut microtome. The first sections of each pair were stained with methylene blue–azure II for morphological study and the second sections were used for demonstration of TRAP activity by a simultaneous coupling method using naphthol AS–BI phosphate as substrate and Fast Garnet GBC salt as coupler [5].

Histopathological evaluation of bone changes was done in sections stained with methylene blue–azure II, on the basis of morphological criteria [6]. Histomorphometric analysis was carried out using an interactive image analyzer (IAS 2000, Delta Sistemi, Rome, Italy). A maximum of 49 different microscopic fields were evaluated at random within the same stained sections, with a ×10 objective.

The following standardized variables were measured in sections stained with methylene blue–azure II [7]:

Osteoclast surface (OcS/BS; %): percentage of trabecular surface in contact with osteoclasts and undergoing resorption.

Osteoclast number (OcN/BS; no./mm^2): number of osteoclasts per square millimeter of trabecular surface.

Moreover, in sections reacted for TRAP cytochemical activity, osteoclasts identified by the TRAP activity were measured as follows:

TRAP-positive osteoclast surface (TRAP+OcS/BS; %): percentage of trabecular surface in contact with TRAP-positive osteoclastic cells.

TRAP-positive osteoclast number (TRAP+OcN/BS; no./mm^2): number of TRAP-positive osteoclastic cells per square millimeter of trabecular surface.

TRAP-positive cells in close association with the calcified bone surface appearing as "mononuclear" or without any nuclei at all in the tissue sections were regarded as osteoclasts and included in the counts.

Serum TRAP activity was measured by spectrophotometric assay, using a reaction mixture containing *p*-nitrophenyl-phosphate as substrate. Intra- and interassay (when calculated over a 60-day period) coefficients of variations were below 3% and 6%, respectively [8].

Comparisons between variables were done by Student's *t*-test for paired data. Correlations were calculated by linear regression analysis on nontransformed data.

Results

The final clinical diagnoses of patients investigated were: idiopathic juvenile osteoporosis (n = 3), involutional osteoporosis (n = 6), established osteoporosis (n = 6), osteomalacia from various causes (mainly malabsorption syndrome; n = 4), primary hyperparathyroidism (n = 2), Whipple's disease (n = 1), non-Hodgkin lymphoma (n = 1), vitamin K acquired deficiency (n = 1). In bone biopsies, the following histopathological features were found: osteoporosis (n = 11), osteomalacia (n = 4), osteoporomalacia (n = 3), osteopenia (n = 3), increased bone turnover consistent with hyperparathyroidism (n = 2), normal bone (n = 1).

Values of the histomorphometric and serum variables considered in this study are reported in Table 1. Values of OcS/BS and OcN/BS were much lower than those of TRAP+OcS/BS (−50%) and TRAP+OcN/BS (−60%), respectively.

Correlations between OcS/BS and TRAP+OcS/BS, and between OcN/BS and TRAP+OcN/BS, were highly significant (Fig. 1). Serum TRAP was weakly correlated with OcS/BS, OcN/BS and TRAP+OcN/BS, respectively (Fig. 2A, B and D). Moreover, serum TRAP was not significantly correlated with TRAP+OcS/BS (Fig. 2C).

Discussion

Until a few years ago, it was generally believed that osteoclasts were giant, multinucleated cells [9]. However, histochemical studies have shown that osteoclasts possess TRAP activity and that some TRAP-positive

Table 1. Values (mean ± SD) of the histomorphometric and biochemical variables

	OcS/BS (%)	OcN/BS (no./mm^2)	TRAP+OcS/BS (%)	TRAP+OcN/BS (no./mm^2)	Serum TRAP (U/l)
	0.79±0.89	0.28±0.35	1.54±1.48	0.71±0.71	15.29±3.39
n.v.	0.18±0.19[a]	0.05±0.05[a]	–	–	10.20±2.10[b]

OcS/BS vs TRAP+OcS/BS: $p<0.001$
OcN/BS vs TRAP+OcN/BS: $p<0.001$

n.v., normal values: [a] Ballanti et al. [6]; [b] Scarnecchia et al. [8].

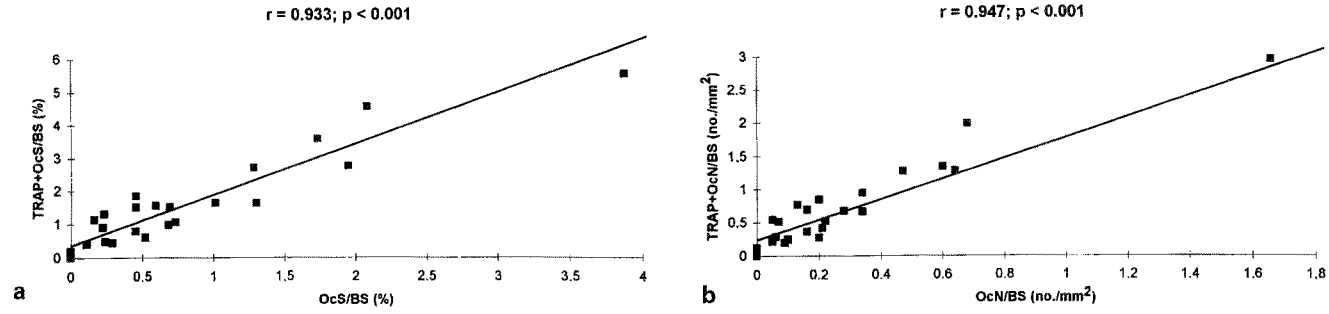

Fig. 1a,b. Correlations between histomorphometric osteoclastic variables measured on the basis of cell shape and nuclearity and on the basis of TRAP cytochemical activity.

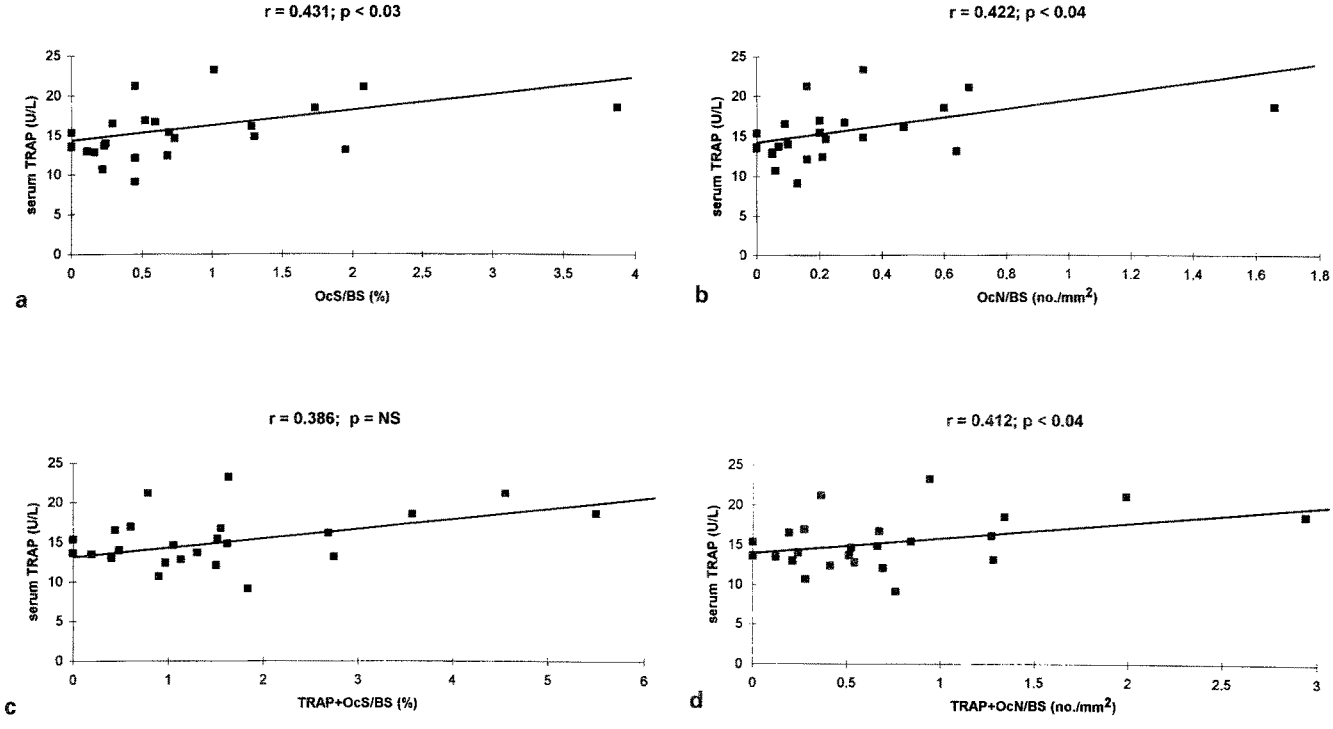

Fig. 2a–d. Correlations between serum TRAP activity and histomorphometric osteoclastic variables measured on the basis of cell shape and nuclearity and on the basis of TRAP cytochemical activity.

mononucleated cells may be found immediately adjacent to bone [2]. These cells may contribute, at least in part, to the bone resorption process, since their adherence to bone is associated with an increased ruffling of their plasma membrane toward the bone surface, similar to the characteristic ruffled border of multinucleated osteoclasts [2]. Recently, the hypothesis has been advanced that these mononucleated cells are

true osteoclasts [10,11], and consequently that evaluation of osteoclastic activity must be based on TRAP expression rather than on multinuclearity [12].

In this respect, it has been hypothesized that the osteoclast count may be greatly underestimated when identification of these cells is based on multinuclearity in standard stained sections [13]. Evans et al. [14] found that 34% of osteoclasts had only one nucleus and that 29% did not show nuclei at all when the sections were stained for acid phosphatase activity. In more detail, using acid phosphatase as a marker and serial sectioning, Kaye [15] demonstrated that in normal bone at least 32% of mononuclear and anuclear osteoclasts were true mononucleated cells, while about 67% of these cells were in fact multinuclear cells in which one or more nuclei were out of the section plane. However, because bone marrow macrophages are normally rich in tartrate-sensitive acid phosphatase, it is possible that those of them which were adjacent to bone surface were counted in these studies [14,15] as mononucleated osteoclasts.

Our study was done on iliac crest biopsies of patients with different pathologies related to bone metabolism, by using TRAP activity as a cytochemical marker of osteoclasts that is more specific than acid phosphatase [2,10]. The use of TRAP as a marker of osteoclasts in tissue sections is firmly established [2,10]. However, no studies, to our knowledge, exist in the literature in which histomorphometric counts of osteoclasts recognized by morphology and by TRAP activity have been systematically compared in human bone biopsies. The results show that OcS/BS and OcN/BS, measured on the basis of cell morphology, are underestimated by 50% and 60%, respectively, in comparison with TRAP+OcS/BS and TRAP+OcN/BS. On the basis of TRAP cytochemical positivity, in fact, not only multinucleated osteoclasts but also elements in direct contact with bone which in the plane of the section appeared as cells showing one nucleus or as small anuclear cytoplasmic fragments, were all identified as osteoclasts and included in the counts. In this respect, serial sectioning, to estimate what proportion of such elements was effectively mononucleated [14], was not done. Although the values of the osteoclastic counts based on morphology are significantly lower than those based on TRAP cytochemistry, correlations between OcS/BS and TRAP+OcS/BS and between OcN/BS and TRAP+OcN/BS are highly significant.

The weak correlations of serum TRAP activity versus OcS/BS, OcN/BS and TRAP+OcN/BS, and the lack of correlation between serum TRAP and TRAP+OcS/BS could be due to several factors. The enzyme activity is not unique to osteoclastic cells, being expressed by cell types other than osteoclasts. Specifically, it has been found that TRAP activity is expressed in osteoblasts and osteocytes located in areas of intense bone remodelling, such as the primary metaphysis of growing bone [5]. The enzyme is also found in activated bone marrow macrophages under certain pathological conditions, such as primary oxalosis [16], chronic granulocytic leukemia and tumor metastasis [17]. Moreover, TRAP activity has been detected in spleen, lung, epidermis, placenta and uterus [18]. The lack of specificity of TRAP activity for the osteoclast is not the only drawback, because the enzyme is unstable in serum, even though serum samples are stored frozen before being assayed. This implies that the spectrophotometric assay should be performed immediately after the serum has been obtained [18]. Finally, the assay may be affected by the presence in the serum of enzyme inhibitors [4]. It is evident that all these factors may limit the ability of the serum enzymatic assay to predict osteoclast activity and that caution is needed in the interpretation of serum data. On the other hand, histomorphometric assessment of cell dimensions (i.e., surface, number) in tissue sections may not necessarily be correlated with cell activity and, with specific reference to this study, with TRAP enzyme activity. Moreover, it cannot be completely excluded that, especially in our patients showing a fairly narrow range of histologically measured osteoclasts, technical variability of bone histomorphometric measurements [7] may contribute, at least in part, to determining the weak correlations of histomorphometric osteoclast variables with serum TRAP activity. In this respect, further studies with a larger range of resorption activity in the subjects and with comparisons between correlations of serum TRAP and parathyroid hormone and other biochemical predictors of bone resorption versus the histomorphometric osteoclast variables could be helpful in assessing the relative sensitivities of different serum markers, for predicting osteoclastic resorption.

Although the present results cannot support the use of TRAP as a conclusively strong biochemical marker of bone resorption, serum TRAP activity has been found to specifically discriminate groups of patients with different metabolic bone diseases [8]. Moreover, its measurement method is simpler and cheaper than other, more sensitive and specific osteoclastic markers [19]. Recently, in an attempt to improve the sensitivity and specificity of TRAP assessment in serum, an immunoassay has been developed using purified enzyme from cord plasma [20]. However, further characterization of the purified enzyme is necessary, with specific reference to its cellular origin. In fact, Price et al. [21] contend that an antibody against placental TRAP that cross-reacts with the osteoclastic enzyme has been raised, rather than an antibody against the osteoclastic enzyme.

With specific reference to the finding that serum TRAP is not or only weakly correlated with the TRAP-positive osteoclastic surface or number, respectively, it might be suggested that the fraction of the osteoclasts which are truly mononucleated are still in the pre-osteoclast phase. Pre-osteoclasts just start to deliver their lysosomal enzymes when, after reaching the bone surface, they spread and adhere to the matrix and increase the ruffling of their membrane [2]. Thus it is possible that the levels of secretory product delivered by these cells are so low that they do not significantly influence serum levels. In this case, serum TRAP levels

would derive mainly from the activity of multinucleated, mature osteoclasts.

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References

1. Palle S, Chappard L, Vico G, Riffat G. Alexandre G. Evaluation of the osteoclastic population in iliac crest biopsies from 36 normal subjects: a histoenzymologic and histomorphometric study. J Bone Miner Res 1989;4:501–6.
2. Baron R, Neff L, Tran Van P, Nefussi JR, Vignery A. Kinetic and cytochemical identification of osteoclast precursors and their differentiation into multinucleated osteoclasts. Am J Pathol 1986;122:363–78.
3. Minkin C. Bone acid phosphatase: tartrate-resistant acid phosphatase as marker of osteoclast function. Calcif Tissue Int 1982;34:285–90.
4. Delmas PD. Biochemical markers of bone turnover. J Bone Miner Res 1993; 8(Suppl 2):S549–55.
5. Bianco P, Ballanti P, Bonucci E. Tartrate-resistant acid phosphatase activity in rat osteoblasts and osteocytes. Calcif Tissue Int 1988;43:167–71.
6. Ballanti P, Della Rocca C, Bonucci E, Milani S, Lo Cascio V, Imbimbo B. Sensitivity of bone histomorphometry in the diagnosis of metabolic bone diseases. Pathol Res Pract 1989;185:786–9.
7. Bonucci E, Ballanti P, Della Rocca C, Milani S, Lo Cascio V, Imbimbo, B. Technical variability of bone histomorphometric measurements. Bone Miner 1990;11:177–86.
8. Scarnecchia L, Minisola S, Pacitti MT, et al. Clinical usefulness of serum tartrate-resistant acid phosphatase activity determination to evaluate bone turnover. Scand J Clin Lab Invest 1991;51:517–24.
9. Mundy GR, Roodman GD. Osteoclast ontogeny and function. In: Peck WA, editor. Bone and mineral research 5. Amsterdam: Elsevier, 1987:209–79.
10. Vaes G. Cellular biology and biochemical mechanism of bone resorption. Clin Orthop 1988;231:239–71.
11. Chambers TJ. The origin of the osteoclast. In: Peck WA, editor. Bone and mineral research 6. Amsterdam: Elsevier, 1989:1–25.
12. Wallach S, Carstens JB, Avioli LV. Calcitonin, osteoclasts, and bone turnover. Calcif Tissue Int 1990;47:388–91.
13. Liu CC, Sherrard DJ, Maloney NA, Howard GA. Reactivation of inhibited bone acid phosphatase and its significance in bone histomorphometry. J Histochem Cytochem 1987;35:1355–63.
14. Evans RA, Dunstan CR, Baylink DJ. Histochemical identification of osteoclasts in undecalcified sections of human bone. Min Electrolyte Metab 1979;2:179–85.
15. Kaye M. When is it an osteoclast? J Clin Pathol 1984;37:398–400.
16. Bianco P, Silvestrini G, Ballanti P, Bonucci E. Paramyxovirus-like nuclear inclusions identical to those of Paget's disease of bone detected in giant cells of primary oxalosis. Virchows Arch A Pathol Anat 1992;421:427–33.
17. Bianco P, Costantini M, Dearden LC, Bonucci E. Expression of tartrate-resistant acid phosphatase in bone marrow macrophages. Bas Appl Histochem 1987;31:433–40.
18. Allen SH, Nuttleman PR, Ketcham CM, Roberts RM. Purification and characterization of human bone tartrate-resistant acid phosphatase. J Bone Miner Res 1989;4:47–55.
19. Rico H, Villa LF. Serum tartrate-resistant acid phosphatase (TRAP) as biochemical marker of bone remodelling. Calcif Tissue Int 1993;52:149–50.
20. Cheung CK, Panesar NS, Haines C, Masarei J. Swaminathan R. Immunoassay of a tartrate-resistant acid phosphatase in serum. Clin Chem 1995;41:679–86.
21. Price CP, Kirwan A, Vader C. Tartrate-resistant acid phosphatase as marker of bone resorption. Clin Chem 1995;41:641–3.

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