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OSTEOCLAST CYTOMORPHOMETRY AND SCANNING ELECTRON MICROSCOPY OF BONE ERODED SURFACES DURING LEUKEMIC DISORDERS.

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Abstract

Tartrate resistant acid phosphatase (TRAP) is a reliable histochemical marker of osteoclasts when used on tissue sections of undecalcified bone. This paper presents an original morphometric analysis which can be done after histochemical identification of osteoclasts. These bone resorbing cells were demonstrated on undecalcified bone biopsies from control subjects and patients presenting a malignant disease of the lymphocyte B lineage. Computerized analysis of the osteoclastic population revealed that: (1) all TRAP positive cells along bone trabeculae belong to a osteoclastic population; (2) that B cell malignancies had an increased bone resorption. At the scanning electron microscopic level small resorption bays (about 10 µm in diameter) were observed either associated or separated from eroded surfaces presenting a normal appearance; TRAP staining of histological sections of undecalcified bone, coupled with morphometric studies, may help in the understanding of bone disease pathobiology.

KEY WORDS: Osteoclast, Tartrate resistant acid phosphatase, cytomorphometry, bone resorption, bone histomorphometry, Scanning electron microscopy.

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Introduction

Bone is subjected to constant remodeling in adults. The osteoclast, the agent of bone resorption, is a large multinucleated cell having the unique capacity to resorb mineralized collagenous matrix. Active osteoclasts are located on bone surfaces and possess membrane specializations identifiable by electron microscopy: the clear (or sealing) zone isolates a subosteoclastic microenvironment wherein the ruffled border actively releases acids and lytic enzymes (see review by Marks and Popoff 1988). Tartrate Resistant Acid Phosphatase (TRAP) is one of the most abundant lysosomal hydrolases in osteoclasts. This enzyme is an iron containing glycoprotein with a molecular weight of about 33,000 (Allen et al. 1989). Its pH optimum is 5.5 at 37°C and the enzyme appears able to hydrolyse a wide variety of substrates: *p* nitrophenylphosphate, di and tri nucleotides (ADP ATP), but not the corresponding monophosphates. Molybdate and fluoride inhibit TRAP, but its hydrolytic activity is not impaired by L(+) sodium tartrate, a competitive inhibitor of prostatic and other lysosomal acid phosphatases. Furthermore, in *in vitro* experiments TRAP was shown to be released in the culture medium by osteoclasts dispersed on devitalized bone (Vaes, 1965). The amount of TRAP activity in the serum of patients with metabolic bone disorders was shown to reflect the bone resorbing activity (Stepan et al. 1983).

Although the exact role of TRAP during bone resorption is still not clearly understood, the enzyme appears to play a key role: molybdate ions or specific antibodies raised against TRAP or uteroferrin (an iron containing protein with an acid phosphatase activity closely related to the TRAP) were recently shown to inhibit TRAP and consequently they abolish the bone resorptive activity of osteoclasts *in vitro* (Zaidi et al., 1989). We have developed a histochemical method based on TRAP staining which allows

histochemical identification and counting of osteoclasts (Chappard et al., 1983b). Briefly, undecalcified bones are embedded in the cold in a glycol and methyl-methacrylate based medium (Chappard et al. 1983a). Sections, 5 µm thick, are incubated with sodium a naphtyl phosphate (the substrate) fast violet B (the diazonium salt) and sodium tartrate. Counterstaining in phosphomolybdic acid - aniline blue provides sharply well defined osteoclasts with a diffuse red brown cytoplasm in close contact with a blue mineralized matrix. Bone marrow cells are unstained (figure 1). This method has been used on more than 1400 human bone biopsies and on animal bone specimens. Counting osteoclast profiles after TRAP staining was shown to be an accurate and highly reproducible method unlike previous ones based on morphological criteria (Evans et al., 1979, Chappard 1990). TRAP is also known as a cytochemical marker for other pathological cells (Gaucher's cells, leukemic hairy cells) but since the enzyme activity is at least 10 times more intense in osteoclasts, these cells never interfere with the osteoclast count. The present paper report two studies making extensive use of cytological morphometry of TRAP stained osteoclasts.

Some malignant diseases of the B lymphocyte lineage are known to be associated with lytic bone lesions and / or hypercalcemia. Although the mechanisms responsible are not yet fully understood, an excessive bone resorption by stimulated osteoclasts has been recognized (Valentin-Opran et al. 1982). Malignant cells exhibit a paracrine secretion of cytokines (mainly Interleukin 1 and Transforming Necrosis Factor ß) that stimulate bone resorption. Lytic bone lesions are a common feature observed in multiple myeloma (the most differentiated B cell malignancy). On the other hand, it is rare for patients with other malignancies of the B cell lineage (BCM) to develop lytic bone lesions (< 3% of the cases). We have recently shown, in a histomorphometric study, that BCM without lytic bone lesion and myeloma patients had a three fold increase of eroded trabecular surfaces. When BCM with lytic bone lesions or hypercalcemia were considered a more pronounced osteoclast resorption was observed with a six fold increase in eroded surfaces (Marcelli et al. 1988; Rossi et al. 1987). Furthermore, the eroded surfaces often had an intriguing appearance in BCM, with minute single bites facing small osteoclasts (micro-resorption). Therefore, the current studies were intended (1) to characterize the osteoclasts facing the eroded surfaces in BCM patients and (2) to compare these eroded surfaces in BCM patients with normal ones at the scanning electron microscopic level.

Materials and Methods

Cytomorphometric analysis

Ten patients with malignancies of the B cell lineage other than multiple myeloma were studied. There were chronic lymphocytic leukemias (5 cases), Waldenström disease (3 cases) and Hairy cell leukemia (2 cases). All patients had a transiliac bone biopsy performed with a trephine method developed in this laboratory. Undecalcified bone cores were embedded in methacrylates and nonserial sections, (7 µm thick) were processed for classical bone morphometry and TRAP staining. Bone biopsies obtained from ten healthy subjects were processed similarly and served as control.

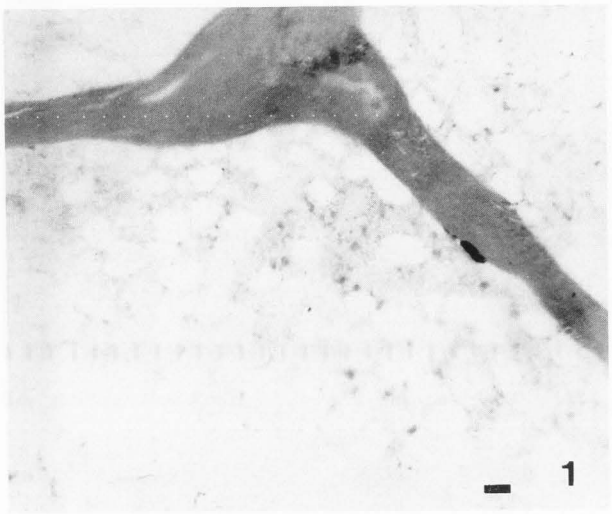
A cytomorphometric study of the length of osteoclast profiles was conducted on a Leitz TAS +; an image analyzer having cabled logics. Special software was designed for measuring each osteoclast profile length (maximum Feret's diameter) at a magnification of 250 (Chappard et al. 1989). Only TRAP positive cells in close contact with trabeculae were considered. Osteoclast segmentation was easily done by thresholding the image based on optical density. Data were stored in an array. Computation and individual profile distribution was provided (with a 5 µm step) for each subject. Pooled results from the 10 patients were compared with those obtained in control subjects. Data were analyzed by the graphical method of Bahr and Mickel (1987) and converted to an arithmetic probability graph.

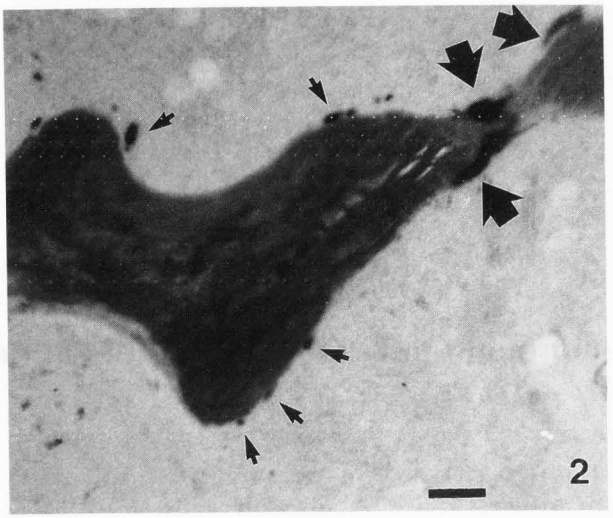
Scanning electron microscopy (SEM)

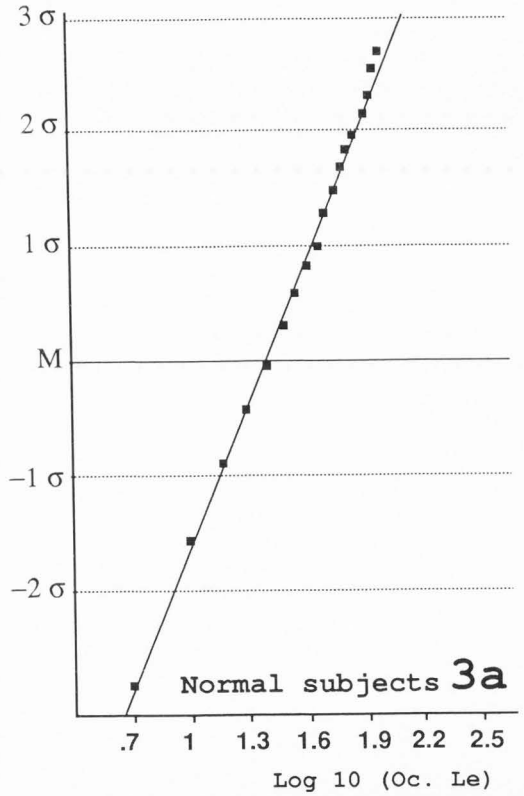
After the pathological examination, plastic was removed from the remnant of the bones cores by dissolving the polymer in dimethylformamide / chloroform (50-50%) for three days at room temperature. Solvents were then removed by two baths of absolute acetone. Bones were carefully rehydrated and nonorganic material (i.e., cells and fibers) was removed with 5% sodium hypochloride for 24 hours (Boyde 1972). Specimens were impregnated with osmium tetroxide, dehydrated and critical point dried with liquid CO_2. A 20 nm gold coat was sputtered for 2 min. Examination was done on a JEOL JSM 35 C at 15 kV accelerating voltage.

Results and Discussion

The light microscopic examination of sections showed that BCM patients had extensively eroded surfaces along trabeculae. Numerous TRAP positive cells were observed in these resorption areas in close contact with bone (Figs. 1, 2). These cells were composed of both normal multinucleated osteoclasts together with an important component of small cells. In controls, the frequency distribution of







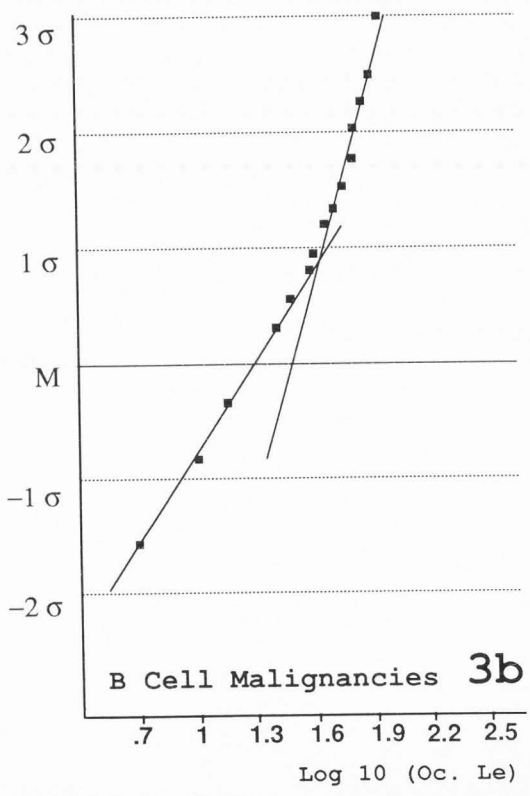


Figure 1: Histochemical staining of tartrate acid phosphatase (TRAP) in osteoclasts. Bone marrow cells are unstained Bar=30 µm.

Figure 2: TRAP staining of a patient with hairy cell leukemia. Note the numerous multinucleated osteoclasts (➨) and the numerous small TRAP positive cells (→) Bar= 30 µm..

Figure 3: a) Graphic analysis of osteoclast length (maximum Feret's diameter) in normal healthy subjects. One straight line indicates a homogeneous cell population.
b) Graphic analysis of osteoclast length in 10 patients suffering from B cell malignancy. Two lines are obtained, indicating the coexistence of two cellular populations.(M = mean, σ = Standard deviation)

osteoclast profile length (Oc.Le) was positively skewed in a fashion that could be described by a lognormal distribution. The mode was centered on the class 20-25

µm. BCM patients presented a bimodal distribution with two clearly separated peaks:one in class 10-15 µm, the second at 20-25 µm. The graphical analysis, after conversion of Log_{10} Oc.Le and plotting with the cumulative frequency, resulted in a straight line connecting the different points in control subjects, thus confirming the log-normalcy of Oc.Le distribution. Cell morphometrists have shown that volume, size, weight, etc. follow a lognormal distribution when an homogeneous cell population is considered (Bahr and Mickel, 1987; Collan, 1987). Log-normalcy is a general biological finding observed in the whole living kingdom and could be related to a geometrical amplification of genes (Sinnott, 1937). In an extensive study of cell volume, Bucher (1955) reported lognormal distributions in 30,000 measurements of human and animal cells. Thus, the lognormal distribution of Oc.Le observed in human controls fits in well with a homogeneous osteoclast population exhibiting a TRAP positivity (Chappard et al. 1989). In BCM, two intersecting lines were clearly observed on the graphical analysis which confirmed the presence of two different cell populations with a similar TRAP stainability (figure 3).

Whether these small TRAP positive cells are mononucleated osteoclast precursors is an open question. With very similar histochemical methods, Baron et al. (1986) and Wijngaert and Burger (1986) were able to identify osteoclast precursors by their TRAP stainability. It is now well established that osteoclast progenitors are mononucleated cells with an extraskeletal origin. However, there is little agreement between authors on the exact stem cell.

Convincing arguments have been repeatedly reported that osteoclasts derive directly from the monocyte / macrophage system (Vaes, 1988; Marks and Popoff 1988). However, several differences between the two cell populations were pointed out because of antigenic determinants and enzymatic techniques. It has been reported that monocytes / macrophages were expressing a tartrate sensitive acid phosphatase isoenzyme of lysosomal origin (Hammarström et al., 1971). Several reports have noted a shift in acid phosphatase secretion when macrophages were stimulated: Dannenberg et al. (1963) showed that alveolar macrophages *in vitro* expressed TRAP positivity; and Razdun et al. (1983) reported that circulating monocytes were lacking TRAP but became TRAP positive when stimulated by a large variety of agents. Yam et al. (1971) and Bianco et al. (1987) reported that bone marrow macrophages expressed TRAP under pathological conditions (chronic granulocytic leukemia, metastatic bone carcinoma...). On the other hand, cultured monocytes whether stimulated *in vitro* by Interferon γ or not, were also found to be TRAP positive (Snipes et al. 1986). Holtrop et al. (1982) reported that mononucleated cells were able to resorb devitalized calcified bone particles implanted in rats but monocytes were also found incapable of degrading bone slices *in vitro* even after long term cultures because no resorption surfaces could be observed at the SEM level (Ali et al. 1984). In the present study, extended eroded surfaces were clearly observed at the SEM level. These resorption surfaces show shallowness and regular bitelike aspect with sharp edges. Orientation of mineralized collagen fibers along resorption surfaces varies according to the degree of resorption of the different lamellae (Boyde, 1972). The shape of each resorption bay was different, a finding in agreement with previous reports (Boyde and Jones, 1979). However, numerous minute and round resorption bays were frequently observed in patients known to have numerous small TRAP positive cells on cytomorphometric analysis (figure 4). These small resorption bays were generally associated with the eroded surfaces exhibiting a normal appearance, but isolated foci were occasionally observed (figure 5).

Whether these abnormally small bays correspond to the small TRAP positive cells or to large osteoclasts with reduced resorbing capacity cannot be resolved by SEM analysis. In future work, we hope to examine and quantify the details of the shape and size of these resorption lacunae both at the light and SEM level. Duncan et al. (1981) have shown that mononucleated cells were frequently observed in the resorption surfaces of patients with rheumatoid arthritis, a chronic inflammatory disease characterized by articular destruction. Mundy (1983) reported that mononucleated cells could be directly responsible for localized bone loss in a number of pathological conditions. The final step (see review by Rossi et al., 1985) in osteoclast differentiation is the fusion of precursors to give multinucleated cells. In BCM this fusion seems to be impaired and the local bone / bone marrow environment and / or the release or lack of cytokines could influence this final step.

Conclusions

Although the exact function of TRAP in osteoclasts have never been elucidated, the enzyme may be playing a key role during resorption of the calcified matrix. TRAP expression by cells of the monocyte / macrophage lineage is modulated by unknown activation processes. Pathological disorders of the B cell lineage induce small TRAP positive cells (mononucleated ?)

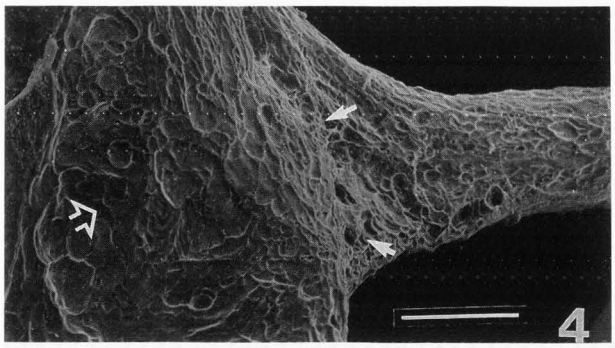


Figure 4: Scanning electron microscope study of the resorption surfaces in B cell malignancies showing both large (⇨) and small resorption bays (➡) Bar = 100 µm.

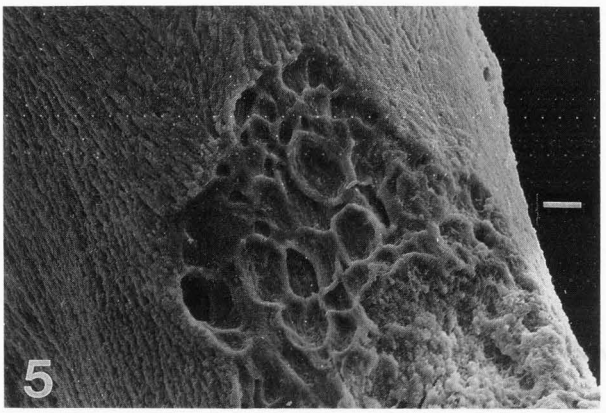


Figure 5: Higher magnification of resorption bays. Bar = 10 µm.

with resorbing properties. TRAP staining provides useful informations on the resorbing activities of bone and may help to elucidate the origin and function of osteoclasts.

Acknowledgments

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Discussion with Reviewers

G.R. Mundy: How can the authors be sure that the mononuclear cells which are TRAP positive are osteoclasts? Is it possible that they are malignant cell ?

Authors: Several cells can express a TRAP positivity in a variety of condition listed hereafter (Chappard 1990)

TRAP POSITIVE CELLS OTHER THAN OSTEOCLASTS
Osteoclast precursors
chick embryo
fetal rat calvaria
experimental *in vivo*
mouse bones
dog alveolar bone
Renal osteodystrophy
Other bone cells:
osteoblasts young rats
osteocytes young rats
Macrophages activated
alveolar macrophages
idiopathic thrombocytopenia
stromal reaction of cancers
Circulating monocytes
minimal activity
enzyme band 4
Cultured monocytes
activated by Inf γ
3 days cultures
U937 enzyme band 4
Blood activated lymphocytes:
infectious mononucleosis
Sézary syndrom
prolymphocytic leukemia
Chronic Lympoid leukemia (rare)
Waldentröm disease (1 case)
Hairy cell leukemia
Neoplastic mast cells
Epithelioid cells:.
Granulomas:sarcoidosis,Hodgkin
Astrocytoma - CNS tumor
Gaucher's cells

Osteoclasts are cells having the highest TRAP content and their cytoplasm give a uniform staining with our method. When other TRAP positive cells are observed within bone marrow, they never interfere with osteoclast count because their TRAP amount is far less than osteoclast (Hairy leukemic cells cannot be taken for osteoclasts although their cytoplasm contain small TRAP positive dots).

S.J. Jones: Serial sections have not been analyzed and there is a possibility of there being more osteoclasts with long cell extensions; and it cannot be excluded from the graphical analysis.

Authors: The mean diameter of the osteoclastic extensions (filipods) is far from the mean diameter of a mononucleated cell and not compatible with the 10-15 µm observed for these small TRAP positive osteoclasts we observed in leukemias. Serial sections have been done and confirm our cytomorphometrical findings obtained with a robust method.