OSTEOCLAST COUNT ON HUMAN BONE BIOPSIES: WHY AND HOW?

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Osteoclasts are multinucleated giant cells which have the capacity to resorb mineralized collagenous matrix (i.e. bone and calcified cartilage). On bone sections, osteoclasts are observed in scalloped areas (so called Howship's lacunae) along trabecular surfaces. Histomorphometric analysis of bone resorption in human osteopathies was first concerned with microradiography, which was said to distinguish active resorption areas but quickly appeared imperfect (25) and of poor value as a routine pathological method. Measurement of total trabecular resorption surfaces (or more precisely "eroded surfaces" according to the ASBMR nomenclature: ES/BS) was then proposed and largely admitted (9,36). Identification of resorption cavities appears rather subtle and only trained pathologists provide reliable and reproducible results. Furthermore, considerable variations for ES/BS in normal subjects have been reported in the literature from different centers (35,36,41,43) and inter observer variations of up to 70% (20) are most probably influenced by subjectivity. Some refinements have been introduced to precise more accurately resorption surfaces with polarized light (47) but ES/BS include both active and inactive resorption surfaces. This may lead to erroneous pathological interpretations in bone diseases associated with an impaired coupling between osteoclastic / blastic activities. For example, osteoblastic activity been drastically decreased in alcoholic osteoporosis, the increase of ES/BS measured in these patients reflects the inability of osteoblasts to fill up the resorbed areas thought osteoclastic activity remains normal.

Why count osteoclasts?

It became apparent to several groups that osteoclast count on bone sections would provide additional informations and could separate inactive from active resorption areas (24). Osteoclast count on sections stained with standard trichromic methods involves an identification based on cytological criteria (i.e. multinucleation, large size, cytoplasmic basophilia...). This method was successfully used to demonstrate the cytoxic effect of ethane 1, hydroxy-1-1 bisphosphonate on pagetic osteoclast (4,5). However, only large and multinucleated osteoclast are recognized and small osteoclast may not be distinguished from the surrounding bone marrow cells leading to underestimation, especially in animals where the number of nuclei is low (1,45). A number of histochemical methods based on enzyme labelling were developed for osteoclast identification since 1958 (10,40). Among all enzymes easily stainable, acid phosphatase (AP) was shown to be the method of choice (38) because quantitative studies had revealed considerable amount in osteoclast - 14 times more than any other bone marrow cell. These former methods identified enzymes on frozen, EDTA decalcified or ground sections; all technics which cannot be used in a routine histomorphometry laboratory. A recent method reported reactivation of AP in bone decalcified by stong acids (33) but demineralization abolishes osteoid stainability. The practical application of plastic embedments at low temperature in methacrylic resins (12,13,15,51) was associated with a considerable developement of enzyme histochemistry. Li and coworkers (31) found in 1970 at least seven distinct isoenzymes of AP separable by polyacrylamide electrophoresis. AP can hydrolyse orthophosphoric monoesters (EC 3.1.3.2). Biochemical studies have demonstrated two distinct AP in bone cells (6,16,32). Osteoblasts were shown to possess a microsomial tartrate sensitive AP (16) while osteoclast possess a tartrate resistant AP (TRAP). The osteoclastic TRAP is probably a glycoprotein with a low molecular weight (32-34000) (29). It is relatively heat stable and its optimum pH
activity is obtained at 37°C, pH 5.5. The enzyme can hydrolyse numerous substrates: paranitrophenyl phosphate, ATP, β glycerophosphate, di and tri nucleotides... The enzyme is activated by ascorbic acid, Fe++, cysteine and dithitreitol. Zn++, Cu++ and molybdate are strong inhibitors. The osteoclastic TRAP migrates as band 5 on acidic acrylamide gel electrophoresis (44) We have reported a routine method for TRAP staining of osteoclasts and up to now, it has been used on more than 1200 human bone biopsies and on bone specimens of rats orbited on the soviet biosatellites (13,14,48). Briefly, undecalcified sections from GMA/MMA embedded bones are incubated in a medium containing sodium α naphthyl phosphate 100 mg, fast violet B 200 mg, L(+) sodium tartrate 15 mg to 0.1 M acetate buffer 100 ml, pH 5.0 for 30 min to one hour. Counterstaining in phosphomolybdc aniline blue and mounting in Apathy's sirup provide sharply well defined osteoclasts (red brown) in close contact with a blue mineralized matrix. Bone marrow cells are unstained (figure 1).

Figure 1: Osteoclasts - TRAP staining - resorbing the mineralized bone matrix (x 300)

Is counting TRAP+ osteoclasts a reliable method?

* In a previous study (48), the inter observer coefficient of variation for osteoclast count was found to be better than 2.2% confirming the reliability and the accuracy of the method.

* Comparison was done between osteoclast identified by morphological criteria and after TRAP staining in 28 patients. Counts of TRAP+ - N.Oc/(B.Ar/T.Ar) - osteoclast profiles are regularly higher than those obtained on Goldner's trichrome stained sections and sometimes up to 3 folds (figure 2). Others have reported 7 to 50 times variations between the two methods for osteoclast count per mm² of section area N.Oc/T.Ar (23). It was also reported that about 30% of TRAP+ osteoclast profiles had no nuclei and these cell fragments are missed when identified morphologically. In spite of the fact that results obtained by the 2 methods are well correlated in our series (r = .76, p < .001), it is likely that several pathological groups of patients might not be recognized as being with a high resorption level specially when numerous small osteoclasts are present. Thus comparison of osteoclast number (N.Oc) in groups of patients with various metabolic bone diseases (and within an heterogenous disease such as osteoporosis) may be biased and erroneous.
Are all TRAP positive cells osteoclasts?

Table 1: TRAP POSITIVE CELLS OTHER THAN OSTEOCLASTS

<table>
<thead>
<tr>
<th>Osteoclast precursors</th>
<th>chick embryo</th>
<th>Kahn et al. (26)</th>
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<tbody>
<tr>
<td></td>
<td>fetal rat calvaria</td>
<td>Ejiri et al. (21)</td>
</tr>
<tr>
<td></td>
<td>experimental (in vivo)</td>
<td>Baron et al. (8)</td>
</tr>
<tr>
<td></td>
<td>mouse bones</td>
<td>Wijngaert et al. (46)</td>
</tr>
<tr>
<td></td>
<td>dog (alveolar bone - tooth eruption)</td>
<td>Marks et al. (34)</td>
</tr>
<tr>
<td></td>
<td>Renal osteodystrophy</td>
<td>Kaye et al. (28)</td>
</tr>
<tr>
<td>Macrophages (activated)</td>
<td>alveolar macrophages</td>
<td>Dannenberg (19)</td>
</tr>
<tr>
<td></td>
<td>idiopathic thrombocytopenia</td>
<td>Yam et al. (52)</td>
</tr>
<tr>
<td></td>
<td>stromal reaction of cancers</td>
<td>&quot;</td>
</tr>
<tr>
<td>Circulating monocytes</td>
<td>minimal activity - enzyme band 4</td>
<td>Snipes et al. (44)</td>
</tr>
<tr>
<td>Cultured monocytes</td>
<td>activated by inf gamma</td>
<td>Weinberg (50)</td>
</tr>
<tr>
<td>U937 (monocytic lineage)</td>
<td>3 days cultures enzyme band 4</td>
<td>Snipes et al. (44)</td>
</tr>
<tr>
<td>Blood activated lymphocytes</td>
<td>infectious mononucleosis</td>
<td>Mover et al.(cf 52)</td>
</tr>
<tr>
<td></td>
<td>Sézary syndrom</td>
<td>&quot;</td>
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<td></td>
<td>prolymphocytic leukemia</td>
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<td></td>
<td>Chronic Lymphoid leukemia</td>
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</tr>
<tr>
<td></td>
<td>Waldenström disease (1 case)</td>
<td>Yam et al. (52)</td>
</tr>
<tr>
<td></td>
<td>Hairy cell leukemia</td>
<td>Li, Yam, Lam (31)</td>
</tr>
<tr>
<td></td>
<td>Neoplastic mast cells</td>
<td>Webb et al. (49)</td>
</tr>
<tr>
<td>Epithelioid c. (Granulomas)</td>
<td>sarcoidosis, Hodgkin</td>
<td>Li, Yam, Lam (31)</td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>(CNS tumor)</td>
<td>Leis et al. (30)</td>
</tr>
<tr>
<td>Gaucher's cells</td>
<td>Spleen and bone marrow</td>
<td>Li, Yam, Lam (31)</td>
</tr>
</tbody>
</table>

Table 1 lists all the different cells expressing a TRAP positivity. Most of them are associated with malignant processes and specially haematological disorders. The TRAP activity in hairy cell leukemia is very strong and it is exploited as a specific marker by haematologists (22). We have examined biopsies from 3 patients with hairy cell leukemia and TRAP+ malignant cells were always observed diffusely within the bone marrow. However, the TRAP activity is at least 10 times more intense in osteoclasts and count is not hampered by neighbouring malignant cells (figure 3). Cells of the monocyte / macrophage lineage also may express a TRAP positivity when activated by pathological processes. Only in a few instances we have observed numerous TRAP+ macrophages within bone marrow
metastatic foci but the TRAP activity of osteoclasts exceeds by far that of these activated macrophages. The main problem concerns osteoclast precursors. Do these cells (which probably belong to the monocyte / macrophage lineage) express TRAP activity? Few observations have been reported in the literature in animals and under experimental conditions or stimulations. Kaye (28) reported TRAP+ mononucleated cells vicinal to osteoclasts in the marrow spaces of patients with renal osteodystrophy and believed they are osteoclast precursors because their number is highly correlated with iPTH levels. In our series, only in a few particular cases (leukemias, Paget's bone disease...) we have observed numerous TRAP+ mononucleated cells and only TRAP+ cells in close contact with the bone matrix must be taken into account for N.Oc measurements.

Figure 3: Bone biopsy from a patient with hairy cell leukemia - TRAP staining; osteoclasts are clearly indentified (↑) and the numerous TRAP positive malignant cells (→) cannot influence measurements.

The size distribution of osteoclast profiles was studied in a series of 10 healthy human volunteers. The mean Feret diameter of osteoclast profiles was measured with an automatic image analyser (Leitz TAS+) and a positively skewed distribution was obtained in a fashion that can be estimated by a lognormal distribution (figure 4a). Data were analysed by the graphical method of Bahr and Mikel (7) and converted to an arithmetic probability graph (figure 4b). A straight line results confirming the lognormality of osteoclast size distribution. Because a cell population have a lognormal distribution of its profile diameter when sectionned (17) we concluded and confirmed that all TRAP+ cells adjacent to the bone matrix belong to an homogeneous osteoclastic population in normal (27). The efficiency of this method was shown in pathological conditions: in cell malignancies of the B lymphocyte lineage, an increased resorption activity is observed and a mononucleated osteoclastic cell population is evidenced together with normal osteoclasts (figure 4c - and abstract this volume). In Paget's bone disease, at least 3 cell populations can be separated (figure 4d)

Figure 4: a) Frequency distribution of the Feret diameter of TRAP positive osteoclasts in normal
b) graphical analysis according to Bahr and Mikel of the Feret diameter distribution of TRAP positive osteoclasts in normal
c) graphical analysis according to Bahr and Mikel of the Feret diameter distribution of TRAP positive osteoclasts in B cell malignancies

d) graphical analysis according to Bahr and Mikel of the Feret diameter distribution of TRAP positive osteoclasts in Pagel’s bone disease

Does the number of osteoclasts reflect their activity at the skeletal level?

Osteoclast number has regularly been reported not to be correlated with osteoclast activity: osteopetrotic rats have numerous TRAP+ osteoclasts which are unable to release the enzyme (42). Also birds have TRAP+ osteoclasts which resorb bone only after oviposition for egg shell calcification (37). On the other hand, Chambers (11) reported that cultured osteoclasts released TRAP when activated and stimulated by calcitropic hormones. In a recent series of 20 patients with various metabolic bone diseases, N.Oc/(B.Ar/T.Ar) was found well correlated with 1-84 iPTH confirming data previously obtained by others (23,28).

In another study of 40 patients we have used a modified hypocalcemia test making use of a single calcitonin injection (18) (abstract from Colson et al., this volume). N.Oc
/(B.Ar/T.Ar) was found well correlated with the urinary calcium and with the slope coefficient of the calcium drop plotted with the square root of time (figure 5).

![Graph](image)

Figure 5: Relationship between osteoclast number and the slope coefficient of the calcitonin test

Is there additional advantages for TRAP staining in pathological practice?

Cancer cells are known to secrete cytokines with OAF properties. Small metastatic foci of neoplastic cells are easily identified as they stimulate osteoclast recruitment in their immediate neighbourhood. Sarcoïd granulomas are detected in the bone marrow (3) because epithelioid cells are slightly TRAP+ (31) and they increase osteoclast number by inappropriate 1-25 OH₂ D3 secretion. Counting osteoclasts is also a very good diagnostic tool for hyperparathyroidism and was found helpful in the survey of osteoporosis treatment by ADFR regimen (2).

How should we measure the osteoclast number?

The simpler way is to count osteoclast profiles in a D2 space. We have developed a program facility (HISTOCLAST) on a flying point microscopic system based on a microcomputer and a digitizing tablet. We recommend to measure each osteoclast length - l(i) - and count the number of fields measured (N) of known area A. All l(i) (from 0 to j) are stored in an array. At the end of measurements, N.Oc/T.Ar = j/(N*A); the mean length of an osteoclast Oc.Le = Σ l(i) / j; the histogram frequency of distribution; the median length of an osteoclast (17) = Σ (mean of class i of the histogram * frequency in i) ... can be provided. Several indexes can be derived from N.Oc/T.Ar: N.Oc/(B.Ar/T.Ar) (bone volume referent) and N.Oc/(B.Pm/T.Ar) (bone perimeter referent) but we found that only N.Oc/(B.Ar/T.Ar) was correlated with biochemical and clinical data. This is in agreement with the modern conceptions of the osteoclast activity in involutional osteoporosis derived from Parfitt's works who observed a disappearance of entire trabeculae by osteoclastic perforations rather than a progressive trabecular thinning (39).

Conclusions:
* Osteoclast count on standard stained sections is an unreliable method and should be avoided. Only TRAP staining provides reliable results.
* In man, TRAP+ osteoclasts form an homogeneous population along the trabeculae and other rare TRAP+ cells (when observed in the bone marrow) never hamper the osteoclast counts.
* The number of osteoclasts N.Oc/(B.Ar/T.Ar) appears to be correlated with osteoclastic activity in human metabolic bone disorders.
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