Histochemical identification of osteoclasts. Review of current methods and reappraisal of a simple procedure for routine diagnosis on undecalcified human iliac bone biopsies

D. Chappard, C. Alexandre, G. Riffat

Laboratoire de Biologie du Tissu Osseux (Pr G. Riffat)
Université de Saint-Etienne, Hôpital Nord, Service de Rhumatologie, 42277 Saint-Priest en Jarez Cedex, France

Key words: Acid phosphatase
Osteoclast
Bone histochemistry
Plastic Embedding
Histopathology
Bone histomorphometry

SUMMARY

Osteoclasts are known to have a high acid phosphatase content. We have adapted the simple simultaneous mono-coupling azo-dye method of Grogg and Pearse to undecalcified bone sections. A cold embedding in a mixture of glycol and methyl methacrylate was shown to well preserve the enzyme activity. Sodium α-naphthyl phosphate (1 mg/ml) and fast violet B (2 mg/ml) are used in 0.1 M acetate buffer, pH 5.0. The addition of 1 mM L(+)-sodium tartrate selectively inhibits the acid phosphoprotein phosphatase ("osteoblastic acid phosphatase") but not osteoclastic lysosomal acid phosphatase. Counterstaining with phosphomolybdic aniline blue WS leads to well contrasted sections, providing accurate measurements of osteoclast number.

RIASSUNTO

È noto che gli osteoclasti hanno un alto contenuto in fosfasasi acida. Gli autori hanno adattato a sezioni di osso non decalcificato il semplice metodo di
copulazione simultanea proposita da Grogg e Pearse. L'inclusione a freddo in una miscela di glicol- e metilmetacrilato risulta in una buona conservazione dell'attività enzimatica. Sodio α-naftil fosfato (1 mg/ml) e fast violet B (2 mg/ml) sono stati usati in tampone acetato 0.1 M a pH 5.0. L'aggiunta di L(+) sodio tartrato 1 mM inibisce selettivamente la fosfatasi acida fosfoproteica ("fosfatasi acida osteoblastica"), mentre non inibisce la fosfatasi acida lisosomale degli osteoclasti. La controcromatizzazione con anilina blu WS fosfomolibdica determina un buon contrasto nelle sezioni, consentendo un'accurata valutazione del numero di osteoclasti.

INTRODUCTION

In the quantitative evaluation of iliac bone biopsies in humans, it is of the utmost importance to be able to precisely assess bone cell dynamics in order to understand skeletal metabolic disorders.

Bone remodeling involves two sequential phases which have been extensively described by Frost (1966) under the BMU theory: a bone resorption phase and a bone formation phase. Bone formation is related to osteoblasts which elaborate osteoid and mineralize it. Tetracycline, used as tissue time marker have helped greatly in studying the dynamics of mineralization (Frost, 1969). Histochimical demonstration of the mineralizing front basophilica with toluidine blue (Bordier, 1973) is also of interest as it is related to the osteoblastic activity (Donitrovsky, 1978). Bone resorption is more difficult to investigate. Meunier et al. (1973) introduced a morphometric measurement of resorption surfaces with an integrating eyepiece. Schenk et al. (1963) measured separately active and inactive resorption surfaces according to the presence of an osteoclast in the lacunae. Osteoclast count per mm² of section area was proposed and applied on undecalcified and stained sections (Alexandre, 1977; Johnell et al., 1977). However, only large and multinucleated osteoclasts are recognized and small osteoclasts may not be distinguished from the surrounding bone marrow cells. Histochimical methods, based on the acid phosphatase labelling, were developed for osteoclast identification since 1958 (Schajowicz and Cabrini, 1958; Burstone, 1958). However, these former techniques requiring frozen sections and freeze dried tissue are inconvenient for routine histopathological diagnosis and for large iliac crest biopsies. Later, osteoclastic acid phosphatase was shown to be preserved in bones demineralized with EDTA and embedded in carbowax (Manning and Butler, 1965) or celloidin (Sams, 1963). Unfortunately, demineralization with EDTA takes a very long time, abolishes the differentiation between osteoid and bone, and removes tetracycline in bone, although Simpson (1981) reported that removal was incomplete in dentine. The method proposed by Wergedal and Baylink (1969) on ground sections cannot be used in a routine histomorphometry laboratory.

Histomorphometry studies of bone need plastic embedments which allow accurate quantitative evaluation of both osteoid amount and bone mineralization rate.

Previous embedding methods are generally incompatible with enzyme histochemistry: epoxies (Xipell and Gladwin, 1972) are usually polymerized at 60°C, methyl methacrylate which was introduced by Burkhardt (1966) is usually polymerized by thermal initiation. Thus, it appears that acid phosphatase activity is lost after prolonged exposure to heat.

We have recently proposed a method for polymerization of large blocks in glycol methacrylate (GMA) at low temperature (Chappard, 1983a) which was then developed and adapted to bone biopsies embedment (Chappard et al., 1983b). Cold embedding was shown to have no effect on osteoclastic acid phosphatase activity. A modification of the Grogg and Pearse monocoupling technique was used and the results are reported here.
MATERIALS AND METHODS

A) Biopsy material. The biopsy material consisted of 180 transiliac bone cores removed from patients with various metabolic bone diseases. All subjects had received a double tetracycline bone labeling. Bone biopsies were performed 1 to 3 days after the last day of the second labeling. They were removed under local anaesthesia with a modified Meunier’s trephine (Meunier et al., 1973; Chappard et al., 1983c) from a standardized point 2 cm below the iliac crest and 2 cm behind the antero-superior iliac spine. The transiliac cores were 8 mm in diameter and ranged from 6 mm to 15 mm in length.

B) Fixation and dehydration. Biopsies were immediately fixed in ice-cold Lillie’s buffered formalin (Lillie, 1954) and kept in the fixative at +4°C for no more than 6 h. They were then dehydrated by immersion for 36 h in absolute acetone (changed 3 times) and then “cleared” by immersion in xylene for 8 h. Dehydration and “clearing” were performed at +4°C with cold reagents.

C) Embedding. Our embedding medium consisted of a mixture of methyl methacrylate (MMA), 300 ml, glycol methacrylate (GMA), 150 ml to which a plasticizer (dibutyl phthalate) 50 ml and a catalyst (benzoyl peroxide) 2.0 g were added. All reagents were obtained from Merck. Both monomers (MMA and GMA) had to be purified before use: hydroquinone (a polymerization inhibitor) was removed from MMA by the method described by Villanueva (1973). Hydroquinone and methacrylic acid (an impurity which strongly binds cationic or thiazine dyes) were removed from GMA by alkali washes and rotatory evaporation under vacuum as previously described (Chappard et al., 1982a; Franklin, 1981).

Bone biopsies were infiltrated over 4 days at −20°C with the embedding medium to which NN dimethylaniline (a polymerization initiator) was added according to our method of “cold embedding” (Chappard, 1983a; Chappard et al., 1983b).

Polymerization was initiated by raising the temperature up to +4°C, so that the redox reaction of benzoyl peroxide degradation by NN dimethylaniline was no longer inhibited and polymerization was able to occur at the same rate in all sites within the biopsy. A water bath was used at the embedding time to refrigerate the moulds and to limit the temperature peak during polymerization.

D) Sectioning. Sections were cut dry at 7 μm using a Jung type K microtome with HK2 or HK3 tungsten knives. Sections for fluorescence study of tetracycline were mounted dried and unstained in neoEntellan (Merck). Sections for routine histomorphometric measurements were stained by free floating in the dye solutions. Sections for histochemical demonstration of acid phosphatase activity were kept dried at +4°C until used.

E) Acid phosphatase - staining procedure. The incubation medium was prepared by adding 100 mg Sodium α-naphthyl phosphate (the substrate), 200 mg. Fast violet B (Gurr, Cl 37265) as the diazonium coupling salt, 15 mg L(+)-sodium tannate to 100 ml of 0.1 M acetate buffer, pH 5.0.

Sections were incubated by free floating in a Coplin jar for 30 min at 37°C. After incubation, all sections were rinsed with tap water and then post-treated in 0.01 M sodium fluoride for 20 min at room temperature. After fresh rinse in tap water, sections were counterstained for 2 min with phosphomolybdic-aniline blue (aniline blue WS, Cl 4275, 1 g, phosphomolybdic acid 2 g, distilled water 100 ml). They are then rinsed twice in tap water.

F) Mounting of sections. Wet sections were flattened and mounted in Apathy syrup.

Basic and Applied Histochemistry, 27, 1983 77
Apathy syrup can be quickly prepared by mixing:

- Hot distilled water (80°C) 50 ml
- D saccharose 50 g
- Extra-pure arabic gum (powder) 50 g
- Thymol 1 crystal

with a mechanical stirrer (2000 rpm at 80°C) for 20 min. At that time, all components are dissolved and the syrup is lacadescent (numerous air bubbles). The bubbles are eliminated by centrifugation (6000 rpm for 15 min). Apathy syrup is now ready for use. It can be stored and dispensed with a 20 ml syringe.

RESULTS

Using 7 μm thick sections, osteoclasts were easily distinguished from other bone or marrow cells: they showed an intense bright reddish brown staining throughout the cytoplasm (Fig. 1) whereas nuclei were not stained at all, and appeared as light holes in the cytoplasm. Multinucleated osteoclasts were in close contact with trabecular bone surfaces of resorption areas. However, in some cases, a few osteoclasts were observed in the marrow spaces, in particular when bone turnover was high as in Paget’s disease of bone (Alexandre, 1977). These likely are newly-formed osteoclasts stemming from the mononuclear stem cell population. They are easily distinct from other giant multinucleated cells such as megakaryocytes which are known not to possess high amounts of lysosomal acid phosphatase.

Osteoblasts and marrow cells as well as the mineralization front were unstained with the acid phosphatase reaction. Also red blood cells (Fig. 2) and siderophages (macrophagic cells containing hemosiderin) were clearly distinct because they remained unstained or were yellowish. We noted that siderophages were present in great number in cases of primary hyperparathyroidism (Fig. 1) and alcoholic cirrhosis of the liver.

Mineralized bone was stained light blue by the counterstain, phosphomolybdic-aniline blue whereas unmineralized bone - or osteoid - remained unstained; also unstained where the cytoplasm of bone marrow cells and muscle fibers. Periosteal mature collagenous fibers were stained blue.

The acid phosphatase staining permitted an accurate count of osteoclasts. For each patient, 4 non-serial sections were processed and counting was done using a standard rectangular photographic eyepiece of known surface, projected over cancellous bone. The number of osteoclasts per mm² of cancellous bone is given by the formula:

\[
N = \frac{n}{S \cdot F}
\]

N = number of osteoclasts per mm² of cancellous bone
n = total number of osteoclasts counted in the 4 sections
S = field area in mm² of the eyepiece
F = total number of fields analyzed in the 4 sections.

The number of osteoclasts per mm² of trabecular bone was found of greatest interest, particularly in osteoporotic patients (unpublished data) because it is related to the trabecular bone volume. Unfortunately, no normal values are available yet.
Fig. 1 - Bone biopsy from an osteoporotic woman. Four osteoclasts are clearly shown in red-brown (arrows). Bone marrow (BM) is unstained (7 μm, x 100).

Fig. 2 - Bone biopsy from a patient with primary hyperparathyroidism. Note the more numerous osteoclasts stained in red-brown (small arrows) easily distinct from siderophages (long arrows) which appear as yellow cells. Mineralized bone (CB) is clearly shown, while osteoid seams (OST ::::), bone marrow (M) and red blood cells (---) are unstained (7 μm, x 100).
DISCUSSION

The method was found highly specific for osteoclastic acid phosphatase. The success and reliability of a reaction for enzymatic osteoclast identification depends of three different factors: a) the method should demonstrate only osteoclastic acid phosphatase and not other enzymes; b) the embedding process should not alter the enzyme activity; and c) the staining conditions should be optimum for the enzyme, i.e.: the incubation should not induce unexpected artifacts.

A) Acid phosphatase - isoenzymes inhibition. Osteoclastic acid phosphatase, a lysosomal enzyme (EC 3.1.3.2) is an orthophosphoric monoester phosphohydrolase. Its activity has been recognized in lysosomes (Doty and Schofield, 1976) as well as outside the cell in the ruffled border and in the Howship's lacunae, immediately underneath the cell (Lucht, 1971; Burstone, 1959). This is generally interpreted as an indication of release of lysosomal enzyme in the resorption zone since other kinds of lysosomal enzymes have also been found between the infoldings of the ruffled border (neutral phosphatases, \( \beta \)-glucuronidase, DNAase) (Doty and Schofield, 1976; Vaes, 1980). It has been demonstrated that a close association exists between bone resorption and the release of lysosomal enzymes in tissue culture (Elion and Raisz, 1978). Although the participation of lysosomal enzymes in the extracellular resorption has been advocated (Vaes, 1980), their capacity to degrade fibrous collagen is limited in vitro. Other additional mechanisms have been proposed: secretion of a neutral procollagenase (Vaes, 1980; Lenaers et al., 1979) or participation of mononuclear phagocytes or fibroblasts secreting collagenase (Heersche, 1978). Furthermore, it has been shown, in a study using immunofluorescent techniques, that osteoclasts of cultured mouse calvaria lack collagenase although other neighbouring cells do contain it (Sakamoto et al., 1978). Nevertheless, the participation of lysosomal enzymes (and especially acid phosphatase) in the intracellular digestion of material taken up by endocytosis into the osteoclast vacuoles is probably one of their most general functions.

Acid phosphatase is also present in bone marrow cells, but quantitative studies on unfixed osteoclasts (Walker, 1972) have shown that the amount of acid phosphatase was 14 times that of bone marrow cells; thus, acid phosphatase appears to be a good marker of this cell. Furthermore, Li et al., (1970), reported the presence of seven electrophoretically distinct acid phosphatases in leukocytes, four of which (isoenzyme 1, 2, 3, 4) are found in normal cells, the other three being associated with several hematological disorders (Gaucher cells, hairy-cell leukemia, acute myeloid leukemia and acute monocytic leukemia). Formaldehyde fixative is a selective inhibitor for isoenzyme 1 and 2, and tartric acid inhibits all isoenzymes. A distinct acid phosphatase is present in red blood cells and is totally inactivated by formaldehyde (Pearse, 1972).

Biochemical studies have demonstrated that there are two distinct acid phosphatases in bone cells (Lieberher et al., 1973; Vreven et al., 1973): the lysosomal enzyme is present in osteoclasts and is also related to \( \beta \)-glycerophosphatase (EC 3.1.3.2); the microsome-linked enzyme present in osteoblasts is related to acid phosphoprotein phosphatase (EC 3.1.3.16).

Previous histochemical studies have shown that acid phosphatase could be detected in osteoclasts but also, to a lesser extent, in osteoblasts, osteoid tissue, and at the mineralization front (Wergedal and Baylink, 1969). Recent studies have shown that osteoblastic acid phosphatase (i.e. acid phosphoprotein phosphatase) is localized in the Golgi apparatus and in the matrix-vesicles released by osteoblasts in the osteoid tissue (Coleman et al., 1980). This enzyme was shown to be markedly inhibited by 1 mM L(+)-sodium tartrate, while lysosomal osteoclastic acid phosphatase is not (Minkin, 1982).
Thus our incubation medium, containing 1 mM L(+)-tartrate, selectively inhibited osteoblastic activity as well as some leucocyte isoenzymes. Inhibition of red blood cell phosphatase activity was produced by the formaldehyde fixation. However, formaldehyde fixation has also been shown to cause a variable loss of activity of other acid phosphatases (Holt and Hick, 1961). Indeed, a short fixation time at +4°C is one of the most suitable fixations usually recommended (Barka and Anderson, 1962; Westen et al., 1981). The importance of dehydration before embedding has been stressed by several authors. Ethanol is known to strongly inactivate acid phosphatase (Gabe, 1968; Barka and Anderson, 1962; Pearse, 1972) and absolute acetone is often recommended as a dehydrating reagent as it causes less inactivation. On the other hand a histochemical method of osteoclasts following dehydration in graded alcohols has recently been reported (Evans et al., 1979; Dunstan and Evans, 1980).

B) Effects of embedding. As extensively reviewed by Gahan (1967), the different classical embedding procedure causes further inactivation of the enzyme. The development of plastic embeddings led us to propose cold embedding procedures with MMA and GMA (Chappard, 1983a) which does not alter the thermolabile enzymatic systems. It was shown that 2h of incubation at 50°C inhibited 25% of the enzyme activity (Lieberherr et al., 1973). A classical paraffin embedding (i.e. at 56°C) may lead to a 95% inhibition (Barka and Anderson, 1962). So excess heat should be carefully avoided during the polymerization steps.

As previously mentioned (Chappard et al., 1982a), the use of hydroquinone-free monomers (pure GMA and MMA) permits very important reductions in the amount of catalyzer. A 0.4% benzoyl peroxide concentration was found the most suitable for bone biopsies. Enzyme activity in tissue sections is known to be enhanced when the amount of this catalyzer is reduced (Westen et al., 1981).

C) Staining methods for demonstrating acid phosphatases. The various techniques for detecting acid phosphatase have been reviewed by Pearse (1972). Briefly, there are 4 types of available methods: a) the lead sulfide method of Gomori, which utilizes sodium glycerophosphate as the substrate in the presence of lead nitrate, is known to be capricious and unreliable. And "practically no applied work on acid phosphatase has been done" with it (Pearse, 1972). Furthermore, lead nitrate is trapped by the calcified components of bone matrix; this method is of no interest in undecalcified sections; b) the indigogenic methods of Seligman (1954), Holt (1958) and Hayashi (1971) using, respectively, sodium indoxyl-phosphate, calcium bromo-indoxylphosphate and acetyl indoxyl-phosphate as a substrate for the enzyme. These methods are not considered to provide exact localization of the enzyme when compared to other ones (Pearse, 1972); c) the post-coupling azo-dye method of Rutenburg and Seligman (1955) uses 6 benzoyl-2-naphthyl phosphate as the substrate during incubation without diazonium salt. The coupling reaction of the dye with the naphthol is performed in a second step. However, this method was shown to possess several disadvantages, such as diffusion of the naphtholic compound during incubation, and the non selective affinity of the substrate for certain tissue components (Gahan, 1967); d) the simultaneous azo-dye coupling methods were introduced in 1949 by Seligman and Manheimer (1949) who mixed together a substrate (calcium α-naphthyl phosphate) and antracquinone-1-diazonium chloride as a diazonium. From their original observations, other methods were developed:

- the phosphate esters of several naphthol AS derivate were developed by Burstone (1958) because of the high degree of insolubility of the naphtholic hydrolysis product;
- Grogg and Pearse (1952) substituted the calcium α-naphthyl phosphate by the more soluble sodium salt and Pearse has listed the suitable diazonium salts as
coupling agents at pH 5.0. The most important problem when using diazonium salts is that the dye is usually soluble in alcohol or xylene so that permanent preparations cannot be made with synthetic mounting media. Therefore, the problem of coupling which is quite insoluble in aqueous or organic solvents was introduced by Barka and Anderson (1962). These methods were recently used in bone histomorphometry to identify osteoclasts (Evans et al., 1979; Dunstan and Evans, 1980). We tried their naphtol AS-TRhexazonium pararosanilin on undecalcified sections from bones embedded in a mixture of glycol and methyl methacrylate and have found some disadvantages: a) only a thin particulate deposit is observed in osteoclasts. This is of no importance in large osteoclasts, but small osteoclasts, as one can observe in some osteopathies (osteoporosis or alcoholic cirrhosis osteopathy (Chappard et al., 1982b) are not recognized. Furthermore, the hexazonium pararosanilin is not totally insoluble in usual solvents (Barka and Anderson, 1962) and mounting may sometimes cause a slight reduction in staining intensity; b) it has been demonstrated that hexazonium pararosanilin does not couple sufficiently fast with the naphtol derivates at pH 5.0 (the optimum pH for acid phosphatase) and that it causes a 10-15% enzyme inactivity at this pH (Barka and Anderson, 1962). Incubation at slightly higher pH (6.5 for example) is recommended but leads to several additional disadvantages (increased inhibition of the enzyme up to 50%); c) when using sodium a-naphtyl phosphate instead of the naphtol AS-TR recommended, a diffuse yellow background staining is observed as hexazonium pararosanilin can couple unspecifically with collagen and other tissue proteins (Barka and Anderson, 1962). When using a toluidine blue counterstain (Evans et al., 1979), abnormal staining patterns are observed.

The method proposed here tends to limit these disadvantages: 1) sodium a-naphtyl phosphate is highly soluble in water; 2) the diazonium salt, fast violet B, does not produce any background staining and couples sufficiently fast at pH 5.0. The azo-dye produced by the enzymatic reaction is diffuse in the cytoplasm of the osteoclasts which are highly stained and no diffusion outside the cells is observed; 3) concentrations of reagents were raised up to 1 mg/ml for the substrate and 2 mg/ml for the diazonium salt. This was found the most suitable concentrations for plastic sections in which the diffusion of reagents is slower than in paraffin or cryostat sections. This was also pointed out by Lojda et al. (1964) and Roudier et al. (1978); 4) addition of Mn** salts (Mn** salts act as activators of acid phosphatase) is not necessary since it presents no additional advantages and it is reported as having no activating effects on bone acid phosphatase (Kirkeby, 1979); 5) the use of an inhibitor of osteoblastic acid phosphatase, 1 mM L(+) sodium tartrate, gives a high specificity to the osteoclastic acid phosphatase staining; 6) the use of phosphomolybdic-aniline blue, a selective collagen stain, gives an extremely sharp contrast with the brown-red staining of osteoclasts. Furthermore, there is no nuclear staining in the bone marrow cells; thus, osteoclast counting is improved and the use of subjective colored filters for light microscopy is not needed; 7) the additional sodium fluoride rinse completely inhibit the remaining enzyme and avoids the undesirable intensification of staining (Hayashi, 1977) and the development of gas bubbles under the coverslip (Pearse, 1972). These two artefacts are due to continued enzyme activity on residual substrate; 8) the mounting in an aqueous medium was found to be superior to air drying after staining (Evans et al., 1979; Westen et al., 1981) as it avoids stretching artefacts in calcified bone. In addition, alcoholic dehydration is not possible with plastic sections containing glycol methacrylate, so the use of hexazonium pararosanilin does not seem absolutely justified; 9) this method is simple and all reagents are inexpensive, which may be of importance for a routine examination of numerous bone biopsies.
In conclusion, this modification of the Grogg and Pearse simultaneous coupling azo-dye method has the advantage of allowing a precise localization of osteoclasts with a simple and inexpensive method.

ACKNOWLEDGEMENT

The authors wish to thank Miss Monzy for typing the manuscript and Mr B. Clerc (Specia Laboratory) for his interest in this study.

REFERENCES


84 Basic and Applied Histochemistry, 27, 1983


