Virchows Archiv A Pathol Anat (1991) 419:425–431

Virchows Archiv A
Pathological Anatomy and Histopathology

Endosteal surfaces in hyperparathyroidism: an enzyme cytochemical study on low-temperature-processed, glycol-methacrylate-embedded bone biopsies

Paolo Bianco and Ermanno Bonucci

Dipartimento di Biopatologia Umana, Sez. Anatomia Patologica, Universita' 'La Sapienza', Viale Regina Elena 324, I-00161 Rome, Italy

Received April 16, 1991 / Received after revision July 8, 1991 / Accepted July 9, 1991

Summary. Alkaline phosphatase (AlP) and tartrate-resistant acid phosphatase (TRAP) activities have been studied comparatively in needle biopsies of the iliac crest of four cases of secondary hyperparathyroidism (renal osteodystrophy). AlP activity was associated with the plasma membrane of osteoblasts and their processes, of reticular cells of bone marrow and of young osteocytes of osteoid borders and woven bone. Moreover, it was detected in the fibroblast-like cells of the endosteal "fibrosis". These cells were orderly in arrangement and were parallel to the endosteal surfaces near zones of bone formation. They were disorderly near zones of bone resorption. A strong TRAP-positive reaction was present in osteoclasts and mononuclear cells of endosteal "fibrosis" and in osteocytes located near active osteoclasts and in woven bone. These results suggest that the so-called fibrosis of hyperparathyroidism, rather than representing reparative, inert tissue, consists of osteoblast-like cells, probably precursors of osteoblasts derived by parathormone-stimulated proliferation of AlP-positive stromal cells of bone marrow, and of TRAP-positive, mononuclear cells, probably preosteoclasts. Moreover, they show that TRAP activity can be present in osteocytes, probably under stimulation by the same factors which stimulate osteoclast activity. The histochemical demonstration of AlP and TRAP facilitates the morphological diagnosis of metabolic bone disease and may improve knowledge of bone physiopathology.

Key words: Hyperparathyroidism – Osteoclast – Osteoblast – Marrow stromal cells – Alkaline phosphatase

Introduction

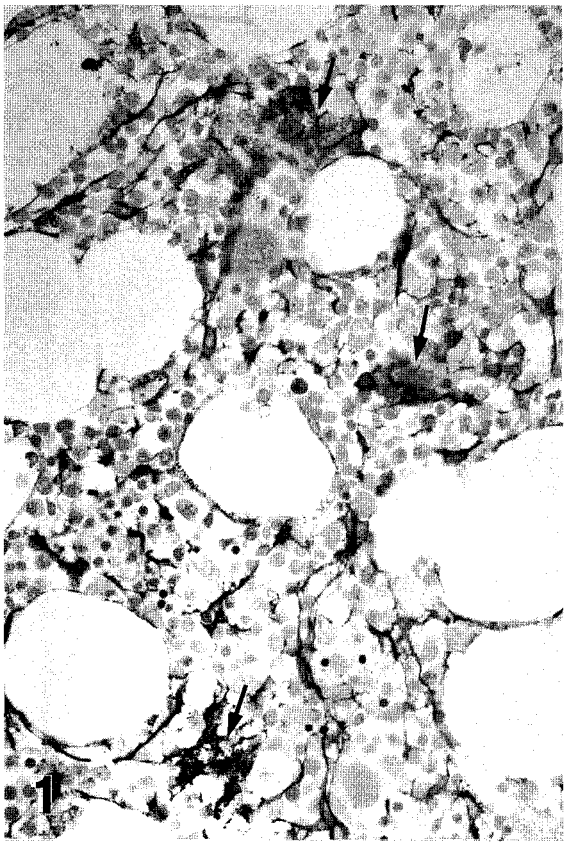
Morphological study of metabolic bone disease over the last decade has relied largely on quantitative methods. While the value of histomorphometry in investigative histopathology of bone is undisputed (Meunier 1983), the attention paid to quantitative features of bone disease has overwhelmed the search for specific qualitative changes which could be demonstrated by the use of cytochemical and/or immunocytochemical techniques. In this regard, bone histopathology has lagged far behind the faster evolution of every other field of histopathology. Undoubtedly, the inherent difficulties related to the special nature of bone as a calcified tissue have played a major role in delaying technological evolution of bone histology.

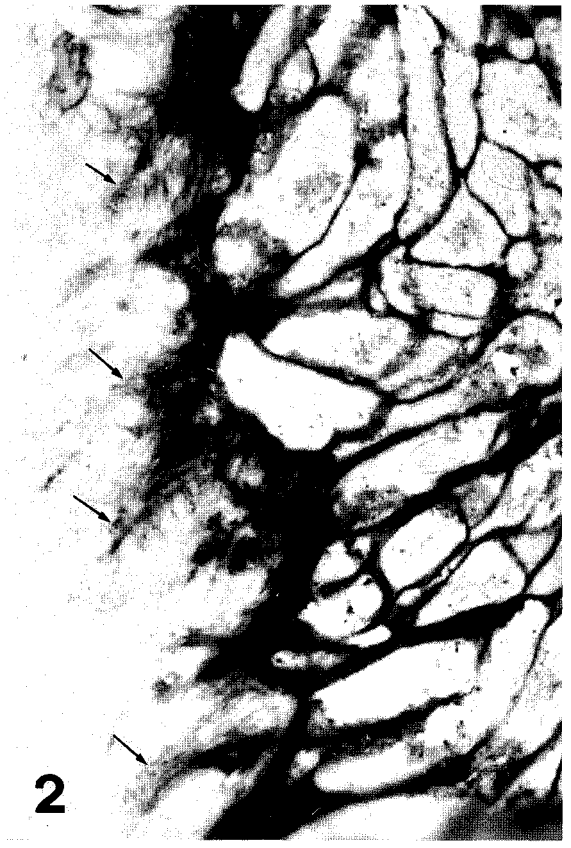
Plastic embedding is widely used for processing bone biopsies. Most laboratories involved in the study of biopsies taken for metabolic bone diseases use methylmethacrylate-based embedding media (Te Velde et al. 1977), which allow sectioning of undecalcified cores but are not well suited for cytochemical studies. In contrast, several methods using glycol-methacrylate (GMA)-based embedding media have been described which allow preservation of enzyme activities in the finished tissue blocks (Ferrell and Beckstead 1990). The main objective in evolving such methods was to apply the enzyme cytochemical techniques used in diagnostic haematology to histological material. These methods have also been applied to the study of problems in bone biology on occasion, but seldom to human material.

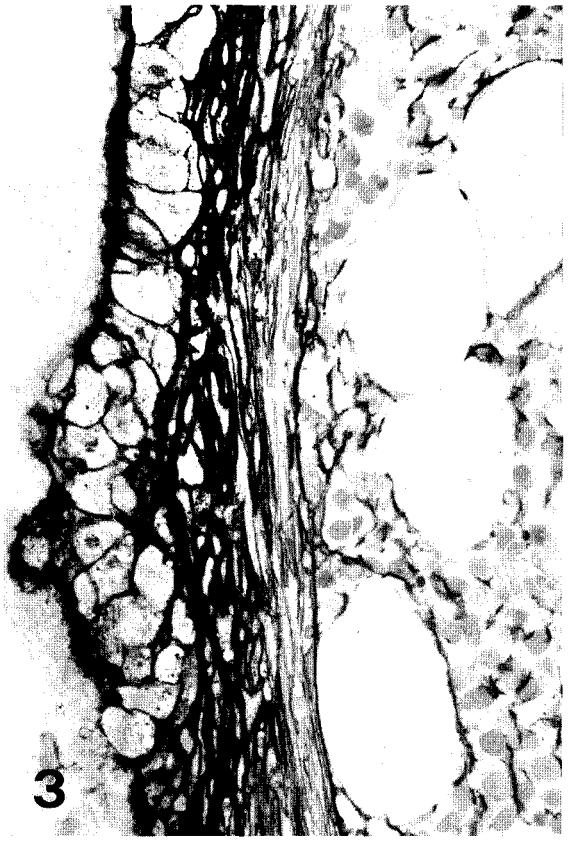
Primary and secondary human hyperparathyroidism have been extensively studied with morphological methods (Avioli 1978; Habener and Potts 1978; Bonucci 1985), including investigations of bone tissue with histology (Jaffe 1972; Malluche and Faugere 1990), histomorphometry (Delling 1987), biophysics (Ascenzi and Marinozzi 1961; Krempien et al. 1972) and electron microscopy (Bonucci et al. 1976, 1978; Delling and Schulz 1978). Histochemical investigations are few and those concerning enzymatic activity and distribution in cells of human bone are practically non-existent.

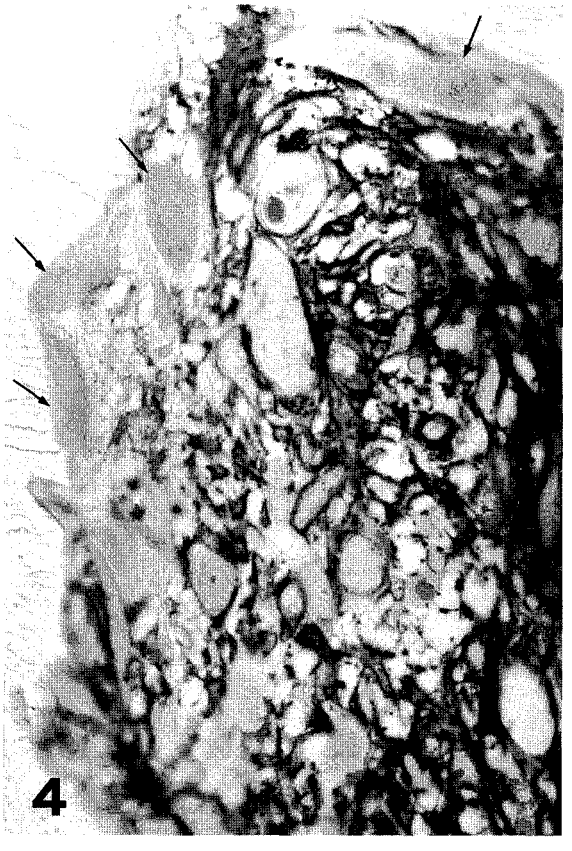
This paper reports an enzyme cytochemical study of bone and bone marrow in secondary hyperparathyroid-

Offprint requests to: P. Bianco


1


2


3


4

ism. Using a technique for preservation of enzyme activities in GMA-embedded human biopsies, some peculiar and previously unrecognized features of these diseases, likely to provide useful information relevant to bone physiopathology, were revealed.

Materials and methods

The investigation was carried out on bone biopsies of four patients with severe renal osteodystrophy of mixed type (secondary hyperparathyroidism due to chronic renal failure). The diagnosis was established on the basis of the clinical and humoral data, and was confirmed by bone histology.

Needle biopsies from the iliac crest were fixed in 4% formaldehyde (freshly made from paraformaldehyde) in 0.1 M phosphate buffer, pH 7.2, for 2 h at 4° C, and successively processed for GMA embedding at low temperature without decalcification, as previously reported (Bianco et al. 1984). Alkaline phosphatase (AlP) and tartrate-resistant acid phosphatase (TRAP) cytochemistry was carried out on semithin (1–2 μm thick) sections, as described by Bianco et al. (1987, 1988b). In some cases, the TRAP reaction was carried out on sections contiguous to those treated for demonstration of AlP activity. Other contiguous sections were stained with haematoxylin and eosin or May-Grunwald Giemsa.

Results

Strong AlP activity was detected in all the cell types in the bone/bone marrow organ shown by previous studies on normal tissues to express significant levels of the enzyme. These include a peculiar cell type in the marrow stroma (reticular cells, Westen and Bainton 1979; Beckstead et al. 1981; Tavassoli and Yoffey 1983) and osteoblasts on appositional bone surfaces. Reticular cells appeared in semi-thin sections as branched cells with elongated thin cell processes, interspersed with haemopoietic cells in marrow spaces (Fig. 1). Osteoblasts appeared as plump cells with AlP reaction product outlining their plasma membrane and their cytoplasmic processes, in agreement with the known association of the enzyme with the cell surface (Figs. 2, 3). In addition, a peculiar finding was the strong reactivity of flat elongated cells comprising the so-called endosteal fibrosis of hyperparathyroidism (Fig. 3). At sites of bone apposition, these cells were regularly aligned at the back side of the osteoblastic row, between this and the marrow space (Fig. 3). Of note was the fact that the flat and aligned AlP-positive cells of the endosteal fibrosis merged on the marrow side with the network of cell processes of AlP-positive reticular cells (Fig. 3).

While routine histology demonstrates a homogeneous appearance of the endosteal fibrosis over the trabecular surfaces, regardless of whether bone formation or resorption is actually taking place, the use of AlP cytochemistry indicated distinct patterns of arrangement of AlP-positive cells as resorptive vs formative sites. The orderly alignment characteristic of formative sites is completely lost at resorptive sites and the AlP-positive cells appear as stellate-to-reticular elements, outlining a meshwork of extracellular spaces (Fig. 4). In these spaces, osteoclasts or mononuclear cells (presumptive pre-osteoclasts) are held.

AlP cytochemistry also demonstrated that expression of the enzyme is retained during the conversion of osteoblasts to osteocytes. Young osteocytes, embedded in newly deposited osteoid tissue and incompletely calcified bone matrix close to the appositional front, displayed AlP activity like osteoblasts, whereas mature osteocytes sitting in lacunae within fully mineralized matrix were negative (Fig. 5).

TRAP activity was demonstrated at all sites of bone resorption, whereas no staining was detected in marrow macrophages. Cells endowed with strong TRAP activity included typical multinuclear osteoclasts and a population of distinctive, small mononuclear cells, not in direct contact with bone surface, but located within a short distance from typical osteoclasts (Fig. 6). Comparison of contiguous sections stained for AlP activity showed that most of the TRAP-positive mononuclear cells were mixed with the AlP-positive, fibroblast-like cells of the endosteal "fibrosis" (Figs. 7, 8). None of the mononuclear cells was identified beyond the limit of "fibrosis" towards the marrow.

Most of the osteocytes did not show TRAP activity. However, most of those located within a short distance from osteoclasts (Fig. 6), and most of those located in woven bone (Figs. 5b, 8), showed variable degree of TRAP positivity ranging from isolated intracytoplasmic granules to diffuse staining. Comparison of contiguous sections showed that most, but not all, of the AlP-positive osteocytes of woven bone were also TRAP-positive (Fig. 5).

Discussion

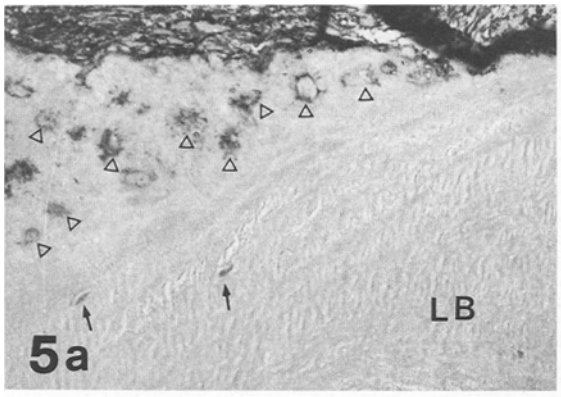
The use of AlP cytochemistry on sections of low-temperature-processed, GMA-embedded biopsies has revealed that the endosteal "fibrosis" of hyperparathyroid bone

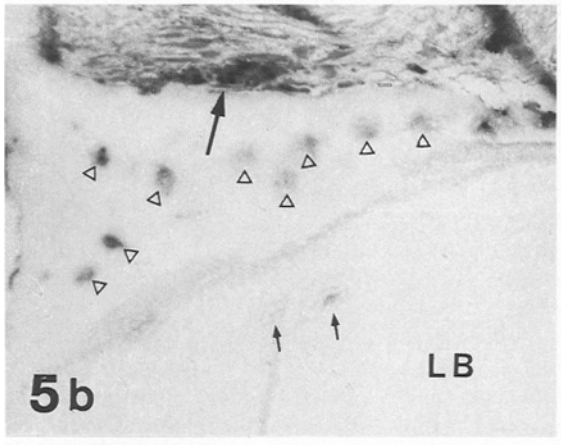
Fig. 1. Alkaline phosphatase (AlP) activity in bone marrow: enzyme reaction is present over thin cytoplasmic processes of reticular cells. *Arrows* point to reticular cells cut tangentially. Non-counterstained, ×300

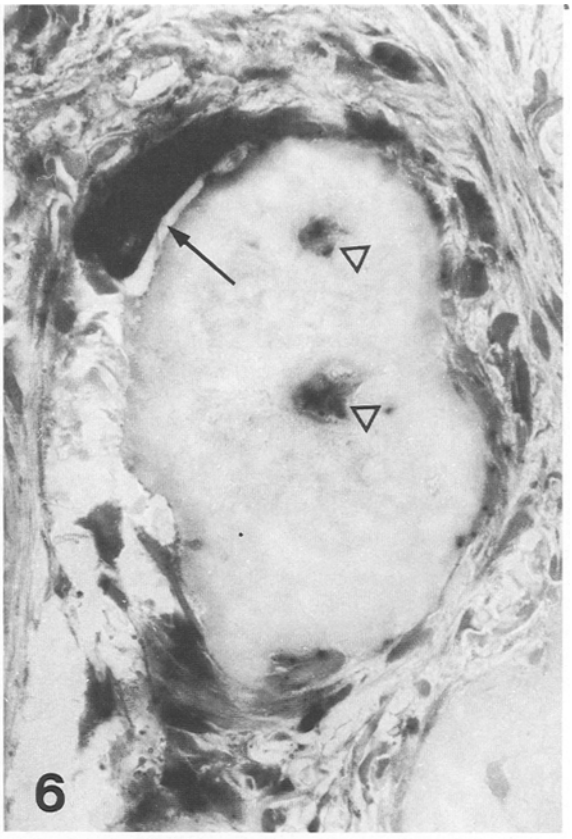
Fig. 2. Detail of AlP activity in osteoblasts: the enzyme activity is associated with the cell membrane and with the cytoplasmic processes (*arrows*) penetrating the bone matrix (*left*). Note positive reaction on the membrane of an osteocyte (*upper left corner*). Non-counterstained, ×750

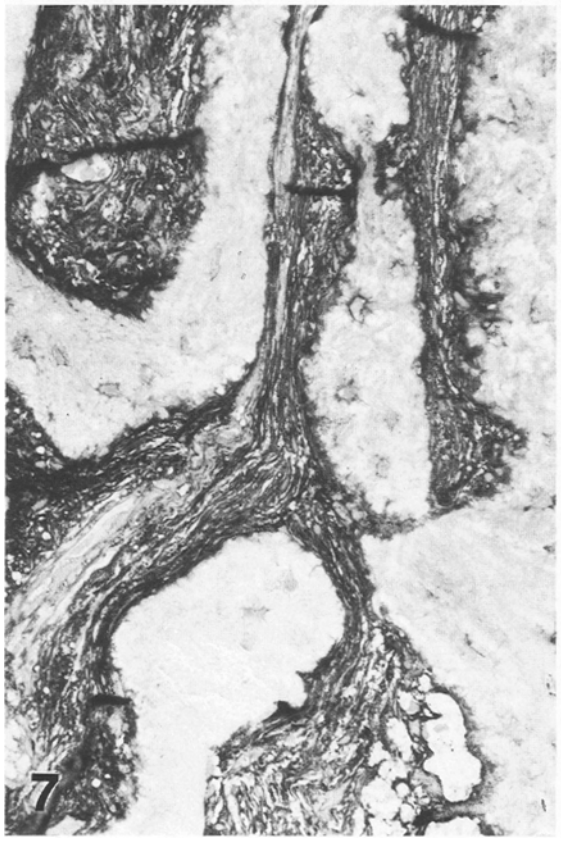
Fig. 3. From left to right: calcified bone matrix, osteoblasts, endosteal "fibrosis", and normal bone marrow. AlP activity is present on the cell membrane of the osteoblasts and of the fibroblast-like cells of the endosteal "fibrosis"; note the regular alignement of these cells and their relationship with the thin, AlP-positive cell processes of bone marrow reticular cells. Non-counterstained, ×375

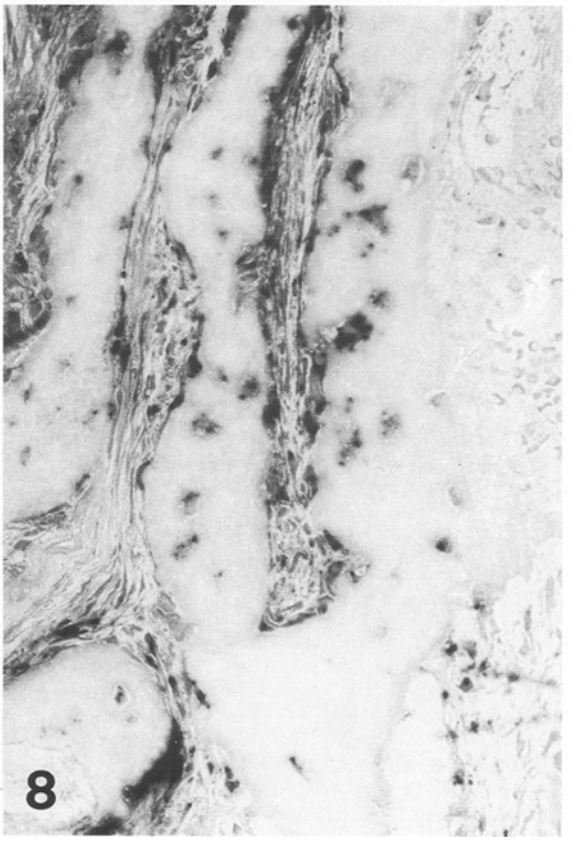
Fig. 4. Area of osteoclastic bone resorption: the AlP-positive fibroblast-like cells form an irregular meshwork. *Arrows* point to active osteoclasts; calcified bone matrix on left. Non-counterstained, ×600


LB
5a


LB
5b


6


7


8

disease, rather than being an accumulation of metabolically spent, reparative fibrous tissue, is a highly cellular tissue made of elongated AlP-positive stromal cells. These cells are arranged in different ways in the "fibrosis" facing formative or resorptive sites along bone trabeculae. At sites of bone apposition, these cells lie parallel to one another and display a high degree of spatial orientation. They merge with plump, active osteoblasts on the bone side, and with AlP-positive marrow reticular cells (Westen and Bainton 1979) on the marrow side. At sites of bone resorption AlP-positive cells of endosteal fibrosis lose their ordered spatial orientation, and define a meshwork of cells and cell processes in which osteoclasts and their putative precursors are held. Thus, the present study reveals a set of major morphological changes which the population of AlP-positive stromal cells undergoes in the vicinities of bone surfaces in hyperparathyroid bone disease. Since these changes are quite unique to this disease, it can be confidently assumed that they are induced by parathormone (PTH).

While the major effect of PTH on bone is an increase in bone resorption, the target cells for PTH are not osteoclasts, which do not bear PTH receptors (Mundy and Roodman 1987). Osteoblasts do bear PTH receptors, and respond to PTH with an increase in cAMP. Jones and Boyde (1976) and Miller et al. (1976) showed that in culture PTH induces changes in shape and orientation in bone cells, and Jones and Boyde (1976) suggested that this could occur by retraction of bone cells and consequent exposure of bone surfaces to osteoclasts. Rodan and Martin (1981) proposed that osteoblast mediate osteoclastic bone resorption in response to stimulation with PTH. Recent studies using ^{131}I-labelled PTH fragments have demonstrated that the cells which bind most of PTH in vivo are not active (plump) osteoblasts directly apposed to bone surface, but a distinct cell lying next to them towards the marrow (Rouleau et al. 1988, 1990). While this cell was considered a distinctive cell type by Rouleau et al. (1988, 1990), its morphology and AlP activity suggest that it corresponds, in the developing marrow, to the AlP-positive stromal cell type described by Westen and Bainton (1979).

Putting together these data and the observations reported in this study, one can speculate that PTH primarily acts on a population of AlP-positive osteogenic stromal cells possibly corresponding to marrow reticular cells. This is consistent with the well-established existence of cells with osteogenic potential in the bone marrow (Friedenstein 1976; Owen 1985). This PTH-responsive stromal cell would thus be the primary player in the pathology of hyperparathyroidism in bone.

TRAP cytochemistry was adopted in this study as a convenient method for identifying osteoclasts. In fact, because of the intense staining, TRAP-positive polynucleated cells (i.e. osteoclasts) appear to be more numerous than they appear to be in contiguous sections stained with haematoxylin and eosin or other histological stains. Consequently, the TRAP reaction is recommended in all cases in which the true degree of active bone resorption must be evaluated.

Previous studies on developing bone indicated that TRAP cytochemistry might also be used as a marker of late osteoclast precursors (Baron et al. 1986; Van de Wijngaert and Burger 1986). Although TRAP activity was more recently proven to lack absolute specificity for osteoclasts (Bianco et al. 1987, 1988a), the small mononuclear cells endowed with strong TRAP activity and located within short distances of resorptive sites are akin to those seen in other instances of increased bone remodelling (Thiele et al. 1989). Although peripheral sectioning of multinuclear osteoclasts may in principle generate images of "pseudomononuclear" cells, this did not appear to be the case in our material, based on the study of multiple, quasi-serial sections. Mononuclear TRAP-positive cells in this study, like similar cells observed in other studies (Baron et al. 1986; Van de Wijngaert and Burger 1986; Thiele et al. 1989), most likely represent "late" mononuclear precursors of osteoclasts. The presence of these cells seems to be an indication that increased bone resorption involves increased recruitment of osteoclast precursors.

The present demonstration of AlP and TRAP activity in osteocytes confirms previous results showing that young osteocytes have AlP activity (Majno and Rouiller 1951), and that TRAP activity can be found in both osteoblasts and osteocytes (Bianco et al. 1988a). Moreover, the observation of TRAP activity in osteocytes located near resorption areas and in woven bone is in agreement with the localization of (tartrate-sensitive) acid phosphatase in cases of increased bone turnover (Gothlin and Ericsson 1973), and with the finding that the activity of the enzyme is inversely proportional to the osteocyte distance from the osteoclasts (Wergedal and Baylink 1969). It is interesting that the same osteocyte can express both AlP and TRAP activity.

Whereas the presence of AlP activity in young osteocytes can be explained as the retention of osteoblastic features in cells that are completing the construction of their lacunae, the presence of TRAP activity cannot easily be explained. Because this enzyme is present in osteoclasts, one might be tempted to speculate that its appearance in osteocytes points to modulation of their function towards osteolysis. Periosteocytic osteolysis has been de-

Fig. 5. Adjacent histological sections reacted for AlP (**a**) and TRAP (**b**) activity: some of the osteocytes (*arrowheads*) in newly formed woven bone show both AlP and tartrate resistant acid phosphatase (TRAP) activities; mature osteocytes (*small arrows*) in adjacent lamellar bone (*LB*) show neither AlP nor TRAP activity. Note AlP activity in cells of endosteal "fibrosis" (**a**, above) and TRAP activity in an osteoclast (**b**, *arrow*). Non-counterstained, × 300

Fig. 6. TRAP activity of osteoclast (*arrow*), osteocytes (*arrowheads*), and several mononucleated cells. Non-counterstained, × 750

Fig. 7. Section adjacent to that shown in Fig. 8: strong AlP activity in cells of endosteal "fibrosis" and on the membrane of several osteocytes. Non-counterstained, × 300

Fig. 8. Section adjacent to that shown in Fig. 7: most of the osteocytes show TRAP activity; several cells of the endosteal "fibrosis" are also positive. Non-counterstained, × 300

scribed several times in human and experimental hyperparathyroidism (Belanger and Robichon 1964; Meunier et al. 1971; Krempien et al. 1973; Bonucci and Gherardi 1977). However, the morphological findings supporting this possibility do not seem to be conclusive (Bonucci 1990) and the concept of osteocytic osteolysis has been challenged (Boyde et al. 1982; Marotti 1990). Because the TRAP-positive osteocytes are located near active osteoclasts, it is possible that both types of cells express TRAP activity when stimulated by the same factor(s). Moreover, the presence of AlP- and TRAP-positive osteocytes in woven bone suggests that, in respect to the elongated osteocytes of lamellar bone, they have not only different morphology (Cane' et al. 1982), but also different functional properties.

The present investigation shows that enzyme cytochemistry of bone cells gives precise, reproducible results which can be used with advantage not only for diagnosis of metabolic bone diseases, but also for investigation of bone physiopathology.

Acknowledglements. This work was supported by grants from the National Research Council and the Ministry for University and Scientific and Technological Research, Italy. The authors are grateful to Silvia Berni for her expert technical assistance.

References

Ascenzi A, Marinozzi V (1961) Biophysical study of von Recklinghausen's disease of bone. Arch Pathol 72:297–309

Avioli LV (1978) Renal osteodystrophy. In: Avioli LV, Krane SM (eds) Metabolic bone disease, vol II. Academic Press, New York, pp 149–215

Baron R, Neff L, Tran Van P, Nefussy JR, Vignery A (1986) Kinetic and cytochemical identification of osteoclast pecursors and their differentiation into multinucleated osteoclasts. Am J Pathol 122:363–373

Beckstead JH, Halverson PS, Ries CA, Bainton DF (1981) Enzyme histochemistry and immunohistochemistry on biopsy specimens of pathologic human bone marrow. Blood 57:1088–1098

Belanger LF, Robichon J (1964) Parathormone-induced osteolysis in dogs. J Bone Joint Surg [Am] 46:1008–1012

Bianco P, Ponzi A, Bonucci E (1984) Basic and 'special' stains for plastic sections in bone marrow histopathology, with special reference to May-Grunwald Giemsa and enzyme histochemistry. Basic Appl Histochem 28:265–279

Bianco P, Costantini M, Dearden LC, Bonucci E (1987) Expression of tartrate resistant acid phosphatase in bone marrow macrophages. Basic Appl Histochem 31:433–440

Bianco P, Ballanti P, Bonucci E (1988a) Tartrate-resistant acid phosphatase activity in rat osteoblasts and osteocytes. Calcif Tissue Int 43:167–171

Bianco P, Costantini M, Dearden LC, Bonucci E (1988b) Alkaline phosphatase positive precursors of adipocytes in the human bone marrow. Br J Haematol 68:401–403

Bonucci E (1985) Morphology of renal osteodystrophy: facts and controversies. In: Norman AW, Schaefer K, Grigoleit HG, Herrath D von (eds) Vitamin D. Chemical, biochemical and clinical update. De Gruyter, Berlin, pp 907–916

Bonucci E (1990) The ultrastructure of the osteocyte. In: Bonucci E, Motta PM (eds) Ultrastructure of skeletal tissues. Kluwer, Boston, pp 223–237

Bonucci E, Gherardi G (1977) Osteocyte ultrastructure in renal osteodystrophy. Virchows Arch [A] 373:213–331

Bonucci E, Gherardi G, Faraggiana T, Mioni G, Cannella G, Castellani A, Maiorca R (1976) Bone changes in hemodialyzed uraemic subjects. Comparative light and electron microscope investigations. Virchows Arch [A] 371:183–198

Bonucci E, Lo Cascio V, Adami S, Cominacini L, Galvanini G, Scuro A (1978) The ultrastructure of bone cells and bone matrix in human primary hyperparathyroidism. Virchows Arch [A] 379:11–23

Boyde A, Jones SJ, Ashford J (1982) Scanning electron microscope observations and the question of possible osteocytic mini-(re)-modelling. In: Silbermann M, Slavkin HC (eds) Current advances in skeletogenesis. Development, biomineralization, mediators and metaoblic bone disease. Excerpta Medica, Amsterdam, pp 305–314

Cane' V, Marotti G, Volpi G, Zaffe D, Palazzini S, Remaggi F, Muglia MA (1982) Size and density of osteocyte lacunae in different regions of long bones. Calcif Tissue Int 34:558–563

Delling G (1987) Bone morphology in primary hpyerparathyroidism – a qualitative and quantitative study of 391 cases. Appl Pathol 5:147–159

Delling G, Schulz A (1978) Histomorphometrische und ultrastructurelle Skelettveränderungen beim primären Hyperparathyreoidismus. Therapiewoche 28:3646–3654

Ferrell LD, Beckstead JH (1990) Plastic embedding. Use of glycolmethacrylate-embedded biopsy material at the light microscopic level. Pathol Annu 25:341–360

Friedenstein AJ (1976) Precursor cells of mechanocytes. Int Rev Cytol 47:327–355

Gothlin G, Ericsson JLE (1973) Fine structural localization of acid phosphomonoesterase in the osteoblasts and osteocytes of fracture callus. Histochemie 35:81–91

Habener JF, Potts JT Jr (1978) Parathyroid physiology and primary hyperparathyroidism. In: Avioli LV, Krane SM (eds) Metabolic bone disease, vol II. Academic Press, New York, pp 1–147

Jaffe HL (1972) Metabolic, degenerative, and inflammatory diseases of bone and joints. Urban and Schwarzenberg, Munich

Jones SJ, Boyde A (1976) Experimental study of changes in osteoblastic shape induced by calcitonin and parathyroid extract in an organ culture system. Cell Tissue Res 169:449–465

Krempien B, Ritz E, Beck U, Keilbach H (1972) Osteopathy in maintenance hemodialysis. Micromorphometric and microradiographic studies with correlations to serum parathyroid hormone and calcitonin levels. Virchows Arch [A] 357:257–274

Krempien B, Geiger G, Ritz E, Buttner S (1973) Osteocytes in chronic uremia. Differential count of osteocytes in human femoral bone. Virchows Arch [A] 360:1–9

Majno G, Rouiller C (1951) Die alkalische Phosphatase in der Biologie des Knochengewebes. Histochemische Untersuchungen. Virchows Arch [A] 321:1–61

Malluche H, Faugere MC (1990) Renal bone disease 1990: an unmet challenge for the nephrologist. Kidney Int 38:193–211

Marotti G (1990) The original contributions of the scanning electron microscope to the knowledge of bone structure. In: Bonucci E, Motta PM (eds) Ultrastructure of skeletal tissues. Kluwer, Boston, pp 19–39

Meunier PJ (1983) Histomorphometry of the skeleton. In: Peck WA (ed) Bone and mineral research annual 1. Excerpta Medica, Amsterdam, pp 191–222

Meunier P, Bernard J, Vignon G (1971) La mesure de l'elargissement periosteocytaire appliquee au diagnostic des hyperparathyroidies. Pathol Biol (Paris) 19:371–378

Miller SS, Wolf AM, Arnaud CD (1976) Bone cells in culture: morphologic transformation by hormones. Science 192:1340–1343

Mundy GR, Roodman GD (1987) Osteoclast ontogeny and function. In: Peck WA (ed) Bone and mineral research annual 5. Elsevier, Amsterdam, pp 209–279

Owen M (1985) Lineage of osteogenic cells and their relationship to the stromal system. In: Peck WA (ed) Bone and mineral research annual 3. Elsevier, Amsterdam, pp 1–25

Rodan GA, Martin TJ (1981) Role of osteoblasts in hormonal control of bone resorption – a hypothesis. Calcif Tissue Int 33:349–351

Rouleau MF, Mitchell J, Goltzman D (1988) In vivo distribution of parathyroid hormone receptors in bone: evidence that a predominant osseous target cell is not the mature osteoblast. Endocrinology 123:187–191

Rouleau MF, Mitchell J, Goltzman D (1990) Characterization of the major parathyroid hormone target cell in the endosteal metaphysis of rat long bones. J Bone Miner Res 5:1043–1053

Tavassoli M, Yoffey JH (1983) Bone marrow: structure and function. Liss, New York

Te Velde J, Burkhardt R, Kleiverda K, Leenheers-Binnendijik L, Sommerfeld W (1977) Methyl-methacrylate as an embedding medium in histopathology. Histopathology 1:319–330

Thiele J, Hoeppner B, Wienhold W, Schneider G, Fischer R, Zankovic R (1989) Osteoclasts and bone remodeling in chronic myeloproliferative disorders. Pathol Res Pract 184:591–599

Van de Wijngaert FP, Burger EH (1986) Demonstration of tartrate-resistant acid phosphatase in undecalcified, glycol methacrylate-embedded mouse bone: a possible marker of (pre)osteoclast identification. J Histochem Cytochem 34:1317–1323

Wergedal JE, Baylink DJ (1969) Distribution of acid and alkaline phosphatase activity in undemineralized sections of the rat tibial diaphysis. J Histochem Cytochem 17:799–806

Westen H, Bainton DF (1979) Alkaline phosphatase positive reticulum cells in the bone marrow stroma. J Exp Med 150:919–937