Journal of Cellular Biochemistry 45:292–302 (1991)

A Role for Osteocalcin in Osteoclast Differentiation

J. Glowacki, C. Rey, M.J. Glimcher, K.A. Cox, and J. Lian

Department of Orthopedic Surgery, Brigham and Women's Hospital (J.G., K.A.C.) and Department of Orthopedic Surgery, Children's Hospital (M.J.G.), Harvard Medical School, Boston, Massachusetts 02115; Laboratoire de Physico-Chimie des Solides, Ecole Nationale Superieure de Chimie, Institut National Polytechnique de Toulouse, Toulouse, France (C.R.); Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01605 (J.L.)

Abstract Specific cellular interactions with components of the extracellular matrix can influence cellular differentiation and development of many tissues. The extracellular matrix of bone is composed of organic constituents and a solid phase of calcium and inorganic phosphate (apatite). When implanted subcutaneously in rats, particles of bone matrix (BPs) recruit progenitors that differentiate into multinucleated cells with osteoclastic features. Because BPs deficient in osteocalcin, a bone matrix protein, were less efficient at promoting osteoclast formation than were normal BPs, we directly examined the influence of osteocalcin on osteoclast differentiation. We evaluated tissue responses to particles of synthetic crystalline apatite alone (Ap), having many of the features of native apatite of mature bone, or to apatite prepared with osteocalcin (Ap/OC), bovine serum albumin (Ap/BSA) or rat bone collagen (Ap/Col). Twelve days after subcutaneous implantation in normal rats, Ap, Ap/BSA, and Ap/Col particles generated a mild foreign body reaction with multinucleated cells in direct contact with the particles; these cells were negative for tartrate-resistant acid phosphatase (TRAP) activity and lacked ruffled borders. In contrast, Ap particles containing approximately 0.1% osteocalcin were partially resorbed and they generated more multinucleated cells that were TRAP-positive, were immunoreactive with an antibody against tartrate-resistant purple acid phosphatase, and displayed ultrastructural features of active osteoclasts including ruffled borders and clear zones. These data support the hypothesis that osteocalcin may function as a matrix signal in the recruitment and differentiation of bone-resorbing cells.

Key words: apatite, bone matrix, foreign body giant cells, implants, extracellular matrix

Osteoclasts are bone-resorbing multinucleated cells that can be identified by their unique cell surface membrane specializations and characteristic morphology [1]. Active osteoclasts attach to bone matrix by an organelle-free clear zone or sealing zone and present a complex ruffled border over the resorption site. Osteoclasts are rich in hydrolytic enzymes and acidification mechanisms [2]. Although the specificity of tartrate-resistant acid phosphatase (TRAP) activity has not been firmly established, it has been used as a marker for active osteoclasts [3]. In addition, osteoclasts possess receptors for calcitonin, which inhibits their resorptive activity [4].

Abbreviations used: Ap, crystalline apatite; BP, bone particle; BSA, bovine serum albumin; OC, osteocalcin; TRAP, tartrate-resistant acid phosphatase.

Received October 19, 1990; accepted October 31, 1990.

Address reprint requests to Julie Glowacki, Orthopedic Research, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115.

Mundy and Roodman [5] have recently reviewed the evidence that the osteoclast arises from the multipotential hematopoietic stem cell, but the precise identities of the osteoclast stem cells and progenitors and their relationship to cells of the giant cell lineage have not been definitively established. Currently, studies on the regulation of the differentiation of osteoclasts concern soluble mediators and cytokines. In contrast, this study focuses attention on the extracellular matrix of bone and its influence upon osteoclastic differentiation.

Implantation of devitalized mineral-containing bone particles (BP) in rats stimulates the recruitment and differentiation of multinucleated cells with many osteoclastic features. These elicited cells resorb the BPs [6], express TRAP activity [7], display ruffled borders overlying the bone substrate undergoing resorption [8], have calcitonin receptors [9], and react with an antibody against rat-bone-purple-acid-phosphatase, with tartrate resistance [10]. Furthermore, re-

sorption of BPs can be inhibited by treating the rats systemically with calcitonin [11] or protamine [12] and is stimulated by heparin [12]. Osteoclasts have many features in common with foreign body giant cells that are typically found in chronic inflammatory tissue reactions. Both are multinucleated and both are formed by the fusion of mononuclear cells of hematopoietic origin. Foreign body giant cells (FBGC) are considered to be macrophage polykaryons [13]. In contrast to bone matrix particles, implantation of particles of nonresorbable plastic (polymethylmethacrylate or polyethylene) elicited FBGCs with features of inflammatory polykaryons [7].

The subcutaneous implantation model of bone resorption has been used to define the effects of alteration of bone matrix components on the resorption process. Bone that was virtually devoid of the vitamin K-dependent protein, osteocalcin, and 93% reduced in the concentration of its characteristic amino acid, γ-carboxyglutamic acid, was obtained from rats treated with warfarin for 6 weeks [14]. Osteocalcin-deficient bone particles were resistant to resorption when implanted subcutaneously in normal rats. The relative resorption was 60% of control bone, as assessed histomorphometrically. In addition, the number of multinucleated cells around those bone particles was reduced by 54%. Mononuclear cells were attracted to control BPs soon after implantation, but cellularity was depressed around osteocalcin-deficient BPs with very few cells within the implant on day 5 (35% of control cellularity) [15]. In implants of normal BPs, TRAP-positive multinucleated cells were evident by day 5, but very few appeared in implants of osteocalcin-deficient BPs even by day 12. The cellularity, TRAP activity, and number of multinucleated cells within osteocalcin-deficient implants not only lagged behind controls but never reached the maximum activity of control BP specimens.

More recently, the use of mixtures of normal and osteocalcin-deficient BPs (with one or the other type prelabeled in vivo with tetracycline) permitted separate analysis of the substrate-specificity of osteoclast recruitment [16]. Those studies revealed that, in the mixed implants, activated osteoclasts were primarily associated with normal BPs and less so with the adjacent osteocalcin-deficient BPs. Those data rule out the possibility that osteocalcin, released from normal BPs in the course of resorption, influences the resorption of adjacent osteocalcin-deficient BPs. The substrate-specificity of the osteocalcin-containing BPs suggests that osteocalcin functions as an extracellular matrix signal in this system and that osteocalcin may have a role in the differentiation or activation of osteoclasts. Because it is theoretically possible that there were other differences, beside osteocalcin content, between the bone particles made from normal rats and the osteocalcin-deficient particles from warfarin-treated rats, we directly examined the influence of osteocalcin on osteoclast differentiation by particles of bone-like, synthetic crystalline apatite, alone or with osteocalcin or BSA.

MATERIALS AND METHODS

Preparation of Particles

Crystalline apatite (Ap) particles were prepared by double decomposition between a calcium nitrate solution [$Ca(NO_3)_2$ $2H_2O$: 17.7 g; H_2O:250 ml] and a diammonium phosphate solution containing sodium bicarbonate [$(NH_4)_2$-HPO_4: 40 g; $NaHCO_3$: 20 g; ammonia solution (d = 0.90): 1 ml; H_2O: 500 ml]. All reagents used were of analytical grade. The pH of the suspension just after mixing was 7.48. The precipitate was left to mature in the mother solution at room temperature for 18 days. The precipitate was filtered and washed with distilled water. The resultant gel was mixed vigorously with purified rat bone osteocalcin (Ap/OC), BSA (Ap/BSA), or collagen (Ap/Col) (0.1% w/w), and the proteins were added as solutions of 5 mg in 1 ml buffer for an estimated 0.1% w/w preparation with 5 g of mineral. This is the approximate concentration of osteocalcin in mature bone. Purified rat bone osteocalcin was prepared from 0.3 N HCl extracts, fractionated by G100 gel filtration, DEAE-52 ion exchange, and C18 reverse phase chromatography [17]. Rat tail tendon collagen was prepared by 0.5 M acetic acid extraction followed by 5% NaCl precipitation, resolubilization in 0.05 M acetic acid, and dialysis against 0.1 M sodium phosphate for precipitation of native collagen fibrils. Bovine serum albumin was obtained from Sigma Chemicals (St. Louis, MO). The dried aggregates of Ap or Ap with proteins were reduced to powders with an agate mortar and sieved to 75–300 μm.

Bone particles (BPs) were prepared from rat long bones (CD strain, Charles River Laboratories) as previously described [18]. In brief, cortical bone was harvested from femoral and tibial diaphyses, cleaned, fragmented, and washed

thoroughly with frequent changes of cold water for 24 hours (500 ml of water per gram of bone). The bone was extracted (200 ml/g) with absolute ethanol over 1 hour and with anhydrous ether over 1 hour. The dehydrated bone was frozen and pulverized in a liquid nitrogen impacting mill (Spex Industries, Metuchen, NJ). The bone particles were seived into a fraction between 75 and 250 μm and stored at room temperature.

Characterization of Apatite Particles

The apatite particles were characterized by chemical analysis, X-ray diffraction, and Fourier-transformed infrared spectroscopy (FT-IR) with deconvolution. Calcium content was determined by atomic absorption spectroscopy [19], and phosphate was determined by colorimetry of phospho-vanado-molybdic acid [20]. Carbonate ions were measured by coulometry (Coulometrics, Inc.) Protein content of the particles was determined on three samples of each preparations. Total protein content was assessed by amino acid analysis after hydrolysis in 6 N HCl for 24 hours at 108°C in vacuo (Beckman 121-M Amino Acid Analyzer), and osteocalcin was determined by radioimmunoassay with species-specific goat anti-rat antiserum and rat osteocalcin standard and tracer prepared in our laboratories [17]. The values were normalized to mg dry weight of the samples ($\bar{X} \pm$ S.D.) X-ray diffraction patterns were obtained on a Debye-Scherrer camera using CuKα radiation or on a curve detector goniometer (Inel CPS 120) using CoKα radiation. Infrared spectra were recorded on FT-IR spectrophotometers (Analect FX 6260 or Perkin Elmer 7700). Deconvolutions were performed with Constructor software.

Implantation

Particulates (50 mg) were implanted bilaterally into subcutaneous pockets in the thoracic area of anesthetized rats (Methoxyflurane, Pittman-Moore, Washington Crossing, NJ). Incisions were closed with a 9 mm wound clip at least 1 cm from the implants [15].

Light Microscopy

Twelve days after implantation, specimens were harvested and fixed in 2% paraformaldehyde, 0.1 M cacodylate buffer, pH 7.4 at 4°C for 6 hours, followed by rinses in 0.1 M cocdylate buffer, pH 7.4, and embedment in glycol methacrylate (JB4, Polysciences Warrington, PA). Three-micron sections were stained for mineral by the von Kossa, H&E technique and for acid phosphatase in the presence of 50 mM sodium tartrate [21].

Particle Resorption

Von Kossa-stained sections were used for histomorphometric evaluation of the particles remaining 12 days after implantation [14]. At 200× magnification with a Zeiss ZIDAS digitizing tablet and drawing arm, the total projected area of remaining particles as percent of the field was determined. Twenty fields were measured for each specimen and the values are expressed as group mean % ± S.D. for 7 rats. Student's "t" test for unpaired data was used to estimate significance.

Microphotometric Measurement of TRAP Intensity

Specimens were cut to 8 μm, mounted, and stained for tartrate-resistant acid phosphatase activity without counterstaining. Staining intensity was measured at 4× magnification with a Nikon Labophot microscope and Nikon P1 microphotometer with Nikon Phoscan-3 software. Transmitted light was measured through 45 to 60 contiguous fields (0.64 mm^2 each) across entire sections and was converted to absorbance per mm^2. For each specimen, the mean for the most intense 30% and the 30th percentile were calculated. Power analysis showed that using the most intense 30% of the total readings conferred statistical validity for group comparison by the Bonferroni adjusted "t" test procedure [22].

Immunohistochemistry

Specimens were fixed in 2% paraformaldehyde, 0.1 M cacodylate buffer, pH 7.4, at 4°C for 6 hours and decalcified for 3 days in 0.03 M sodium citrate and 0.05 M formic acid. Paraffin-embedded sections were cut at 5 μm and stained for immunoreactivity with anti-purple acid phosphatase IgG (1:10,000) provided by S. Toverud (University of North Carolina) and according to published procedures [23]. Reactivity was visualized by 3 methods: 1) rhodamine-conjugated goat anti-rabbit IgG as secondary antibody (Capel, West Chester, PA), 2) the peroxidase-antiperoxidase and 3-amino-9-ethyl carbazol technique (Pel-Freeze, Rogers, AR), and 3) silver-enhanced Auroprobe (Jansen, Oler, Belgium), according to the manufacturers' directions. Con-

trols included omission of primary or secondary antibody.

Transmission Electron Microscopy

For electron microscopy, specimens were minced into 1 mm^3 pieces, fixed for 3 hours in 4.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, post-fixed for 3 hours in 1% osmium tetroxide in 0.1 M cacodylate, pH 7.4, and embedded in Spurr's resin (EMS, Fort Washington, PA). Sections were cut at 0.05 μm and stained with uranyl acetate and lead citrate for viewing on a Philips 300 transmission electron microscope.

RESULTS

The synthetic apatites were characterized by chemical analysis, X-ray diffraction, and by resolution-enhanced Fourier transformed infrared spectroscopy. The X-ray diffraction patterns were characteristic of poorly crystalline apatite and very similar to that of bone mineral. Figure 1 shows a 002 band, a broad mixed reflection corresponding to unresolved 211, 112, 300, and 202 bands, and faint bands at 310, 222, 213, and 004, characteristic of the apatitic structure. The relatively sharp 002 and 004 bands suggest that individual crystals are, as in bone mineral, elongated along the c-axis of the hexagonal cell [24,25]. In order to reveal fine details related to the short-range organization of the mineral ions, the synthetic materials were examined by FT-IR spectroscopy (Fig. 2). The ν_2 CO_3 domain showed 2 main bands at 879 and 871 cm^{-1} which have been assigned to carbonate located, respectively, on monovalent anionic sites of the apatitic structure (OH sites in type A carbonate) and on trivalent anionic sites (PO_4^{-3} sites in type B carbonate) [26]. The intensity ratio of these bands is identical with that found in mature bone mineral [27]. A third carbonate band in the shoulder at 866 cm^{-1} corresponding to a labile carbonate ion may be observed in the spectrum but with a weaker intensity in synthetic crystals than in mature bone. The ν_4 PO_4 domain exhibits phosphate bands observed in natural, biologic, and synthetic apatites. As in bone, a small shoulder also appears at 610–615 cm^{-1}, which has been assigned to a non-apatitic, labile phosphate group. In all cases, incorporation of protein (osteocalcin or bovine serum albumin) in the samples did not modify the main structural characteristics of the apatite crystals.

Chemical analysis of the synthetic apatite's major constituents (Ca, 33.9%; PO_4, 53.3%; CO_3, 4.6%, by weight) was consistent with the mineral composition of mature bone with a slightly lower carbonate content. The calcium-to-phosphate atomic ratio was 1.51, compared to 1.51 to 1.65 in rat bone [28]. The stoichiometry of the synthetic apatite mineral, expressed as Ca/(P + CO_3) ratio (carbonate substitutes principally for phosphate in these crystals) is similar in the synthetic crystals (1.33) and in bone mineral (1.38–1.39) [28]. Osteocalcin concentration in the nominal 0.1% (w/w) preparations ranged between 0.06 and 0.08% (w/w) by radioimmunoassay of 3 lots. A very small deviation was observed in triplicate samples of each lot (< 5%) and suggests uniform distribution of osteocalcin. Replicates of samples containing lower protein concentrations were too variable to indicate uniform distribution (data not shown). Thus, with this crystalline procedure, it was not possi-

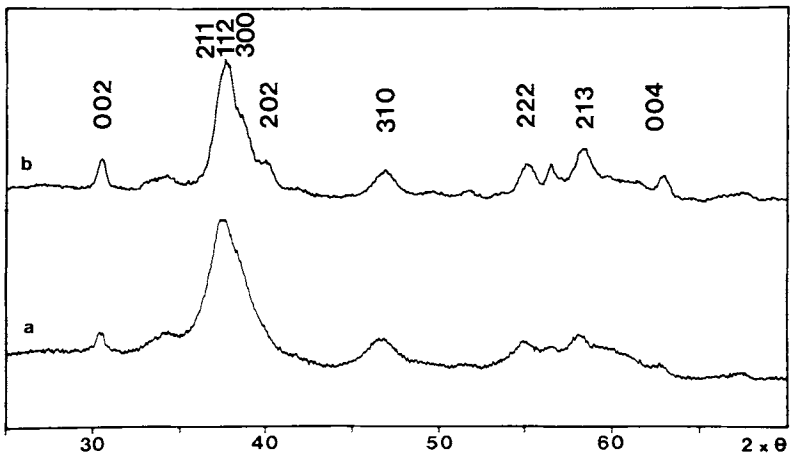

Fig. 1. X-ray diffractometry of (a) rat bone mineral (b) synthetic apatite-containing osteocalcin.

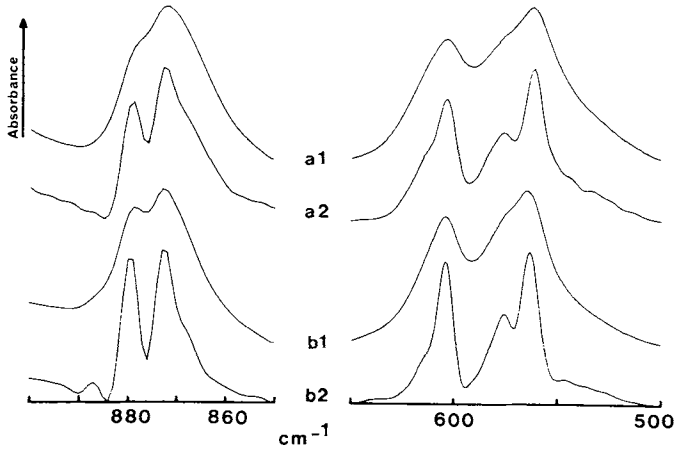

Fig. 2. FT-IR Spectra in the ν_2 CO_3 and ν_4 PO_4 domains. **a:** Rat bone mineral. **a1:** Raw spectrum, without deconvolution. **a2:** After deconvolution, resolution-enhanced spectrum (σ = 8 cm^{-1}, k = 2.5 for ν_2 CO_3; σ = 18 cm^{-1}, k = 2.0 for ν_4 PO_4). **b:** Synthetic apatite-containing osteocalcin. **b1:** Raw spectrum without deconvolution. **b2:** Resolution-enhanced spectrum (same conditions as a2).

ble to test other doses with confidence. Furthermore, as pointed out by Hauschka and Wians [29], it is likely that not all sufaces on a hydroxyapatite crystal present equivalent inter-calcium 2+ intervals for uniform binding with osteocalcin. Thus, with unsaturating concentrations of osteocalcin, lack of homogenous distribution was not unexpected. Albumin concentration was uniform throughout each preparation and was measured as 0.16% protein concentration for the nominal 0.1% (w/w) preparation.

Apatite particles alone (Ap) or those containing bovine serum albumin (Ap/BSA) or collagen (Ap/Col) generated a mild inflammatory reaction with multinucleated cells in direct contact with the smooth edges of the particles, with no evidence of resorption in day 12 specimens (Fig. 3a). In contrast, Ap/OC particles were penetrated by multinucleated cells (Fig. 3b). In each group, tissue between the particles was composed of fibrous tissue with evenly distributed small blood vessels. At the time of implantation, the individual particles and the implants were the same size. Histomorphometric analysis of the particles remaining 12 days after implantation demonstrated greater resorption of the Ap/OC particles. The Ap/OC particles were smaller and represented 44.85 ± 1.94% of the projected area of the tissue in which they were located. The Ap particles occupied 51.03 ± 1.06% of the area of the tissue in which they were located. This 12% difference ($P < .005$) reflects the resorption of the osteocalcin-containing particles over 12 days.

Histochemical localization of tartrate-resistant acid phosphatase (TRAP) activity showed

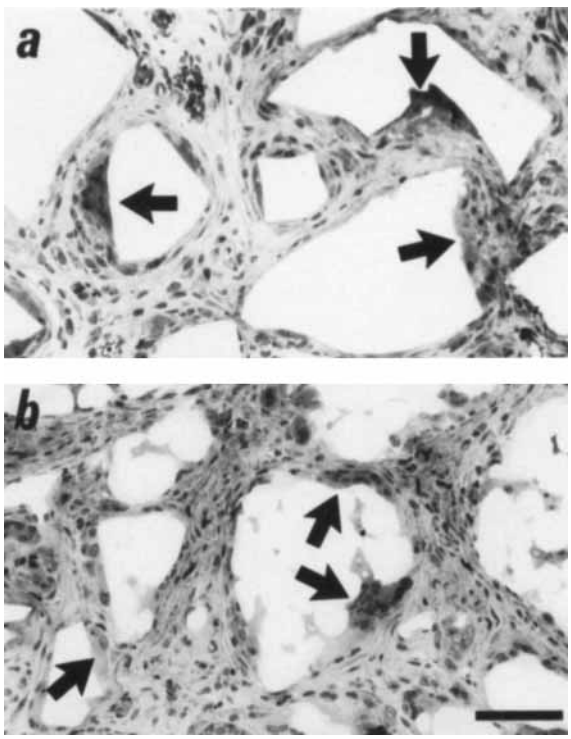

Fig. 3. **a:** Light micrograph of Ap particles and elicited tissue 12 days after implantation. **b:** Micrograph of Ap/OC particles and elicited tissue 12 days after implantation. Arrows indicate multinucleated cells. Particles show evidence of resorption and cellular penetration. Toluidine blue. ×125 magnification.

positive multinucleated cells associated with Ap/OC particles. These cells were similar in intensity but not in numbers to cells elicited by particles of rat bone. There was no TRAP activity in cells generated by 3 lots of Ap particles, 2 lots of Ap/Col particles, and 2 lots of Ap/BSA

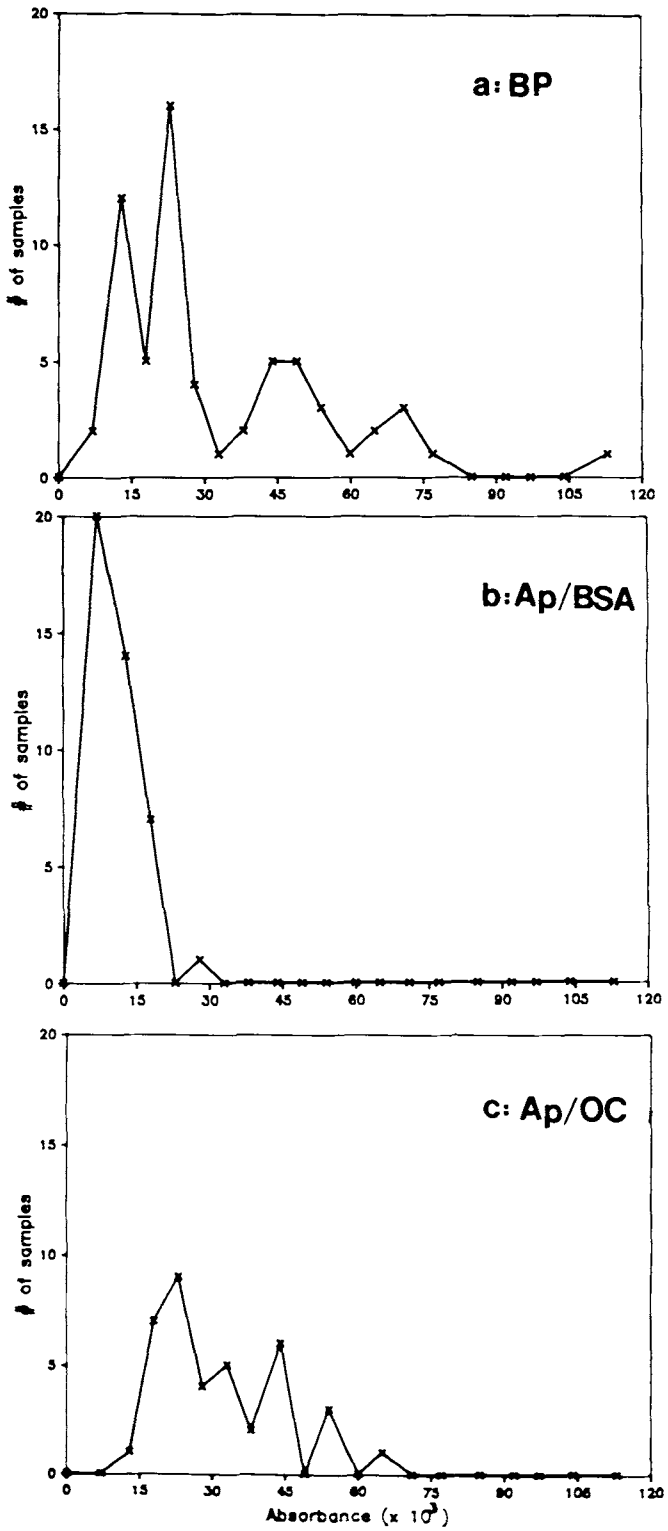

Fig. 4. Frequency distribution of the number of fields (samples) with specific absorbance values in one representative, TRAP-stained 8 μm section of tissue recovered 12 days after implantation of (a) bone particles (BP), (b) apatite with BSA (Ap/BSA), or (c) apatite with osteocalcin (Ap/OC). Two of three lots of Ap/BSA were negative for TRAP activity. The sample shown is from one lot that generated cells with weak reactivity.

TABLE I. Tartrate-Resistant Acid Phosphatase Activity in Tissue Elicited by Particles†

Particles	N	Absorbance × 10^{-3} per mm^2	
		$\bar{X}_{30}$	30th %-ile
BP	9	56.4 ± 14.2	44.8 ± 10.2
Ap	12	19.9 ± 6.1	15.5 ± 3.8
Ap/BSA	11	20.5 ± 5.7	15.9 ± 4.9
Ap/OC	12	30.6 ± 15.2*	23.9 ± 11.9*

†N is the No. of implants and separate histograms (illustrated in Fig. 4).
*Significantly different from Ap and Ap/BSA $P < 0.05$.

particles. However, one lot of Ap/BSA particles demonstrated a small amount of weak histochemical reactivity in elicited tissue. In order to quantitate relative enzyme activity in cells recruited by that lot of Ap/BSA particles, by Ap/OC and Ap particles, and by rat bone particles (BP), we developed a microphotometric method for quantification of staining intensity in histological sections of elicited tissue. Each section generated a frequency distribution of absorbance for approximately 40 to 60 microscopic fields (one from each group shown in Fig. 4). Because of skewing of readings toward background, especially in the Ap and Ap/BSA groups, data are presented in Table I as the group mean for the average of the 30% most intense values and as the group mean of the 30th percentile values, after conversion to absorbance. The absorbance for Ap particles represents the baseline for this method. The data demonstrate less TRAP activity in the foreign body giant cells associated with Ap or Ap/BSA particles than the osteoclastic cells associated with BPs ($P < 0.001$) and demonstrate significantly more TRAP activity by cells recruited by Ap or Ap/OC particles than by Ap/BSA particles ($P < 0.05$).

Antibody against rat bone purple acid phosphatase identified the multinucleated cells that were associated with osteocalcin-containing particles (Fig. 5). This specificity was demonstrable by rhodamine, peroxidase, and the silver-enhanced gold techniques. Controls without primary antibody or with non-immune rabbit IgG did not stain. Multinucleated cells associated with Ap particles did not display immunoreactivity.

Ultrastructural characterization of the various multinucleated cell showed typical foreign body giant cells associated with Ap or Ap/BSA particles (Fig. 6a). The cells were large and multinucleated, frequently with a peripheral distribution of nuclei. The cells were closely associated with the smooth surfaces of the mineral. The face of the giant cell not in contact with the particles was characterized by highly convoluted membrane foldings and irregular microvilli of variable lengths. These features are typical for Langhans-type of macrophage polykaryons or foreign body giant cells. In contrast, the multinucleated cells elicited by Ap/OC particles showed many of the features of resorbing osteoclasts (Fig. 6b). They were rich with mitochondria and their cytoplasm penetrated into the particles. They frequently had clear zones of attachment to the substrate and membrane specializations similar to the ruffled borders of in osso osteoclasts and of BP-elicited osteoclasts [8] (Fig. 7).

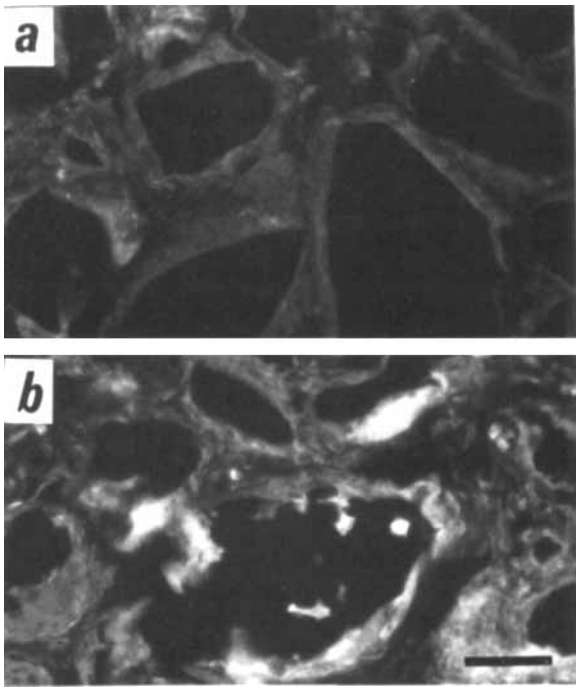

Fig. 5. Indirect immunofluorescence visualization of tartrate-resistant purple acid phosphatase in paraffin sections, demonstrated by rhodamine-conjugated second antibody. **a:** Ap particles. **b:** Ap/OC particles. ×125 magnification.

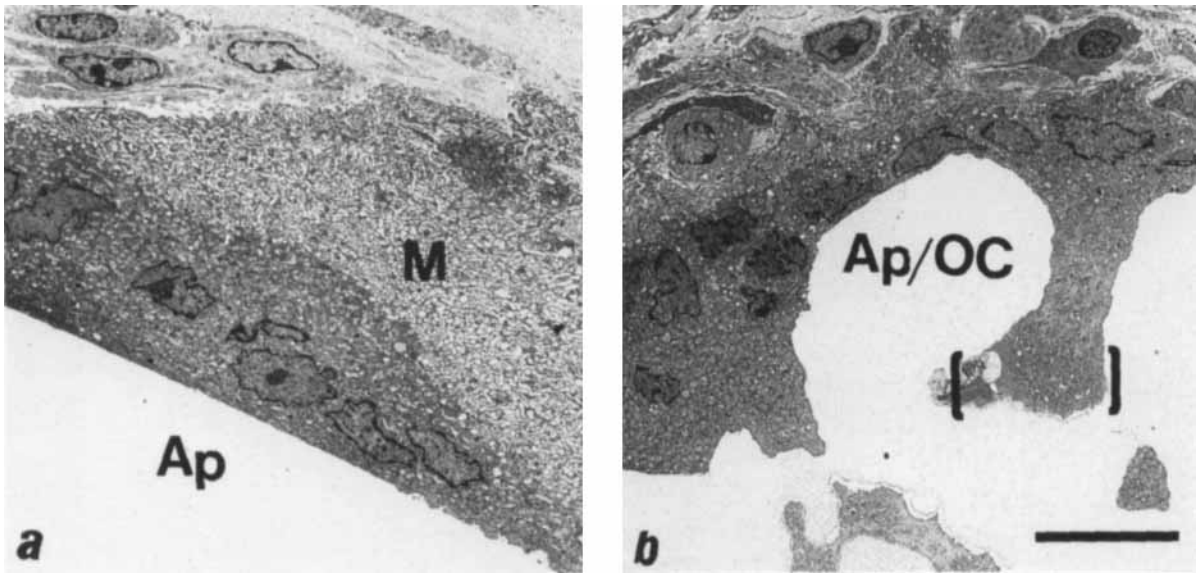

Fig. 6. **a:** Transmission electron micrograph showing multinucleated cell adherent to Ap surface 12 days after implantation. The face of the cell not in contact with the particle has highly convoluted microvilli (M). **b:** Transmission electron micrograph showing partially resorbed Ap/OC particles and associated multinucleated cell 12 days after implantation. Box indicates region magnified in Figure 7d. ×2,065 magnification.

DISCUSSION

Because of the impaired resorption of osteocalcin-deficient bone particles, we speculated that osteocalcin may play roles in osteoclast differentiation and in skeletal resorption [14,15]. Other studies suggested that osteocalcin in normal bone functioned as an insoluble substrate signal in the extracellular matrix [16]. In this study, osteocalcin-containing apatite particles reproducibly promoted the differentiation of multinucleated resorbing cells with features characteristic of osteoclasts.

The use of synthetic and well-characterized particles of apatite permits the assessment of the role of incorporated factors on the phenotype of cells elicited by the particles. Commercial materials offered as hydroxyapatite have a wide range of physical and chemical properties [30]. The crystalline apatite particles prepared for this study were demonstrated to be very similar to mature bone mineral in composition and structure, as judged by X-ray diffractometry and FT-IR spectral analysis. These features allowed us to evaluate the tissue responses to bone-like mineral with and without the bone matrix protein, osteocalcin. It is possible, however, that the synthetic particles may not exactly represent the distribution or orientation of osteocalcin as in mature bone matrix. Mature bone matrix is characterized by a large surface area of small apatite crystals that are available for exchange with body fluids. The distribution of osteocalcin in the synthetic crystals and its similarity to the distribution of osteocalcin in the porous structure of bone with its constituent organic components are not known. Difference in the availability or uniformity of osteocalcin throughout the matrix may account for the differences in the degree of bioactivity observed between Ap/OC and BP matrices. It is also likely that other organic constituents of bone matrix contribute to the osteoclastogenic property of bone matrix particles. Nevertheless these studies show that matrices of bone-like apatite, alone or containing collagen or BSA, do not recruit osteoclasts and that matrices of osteocalcin and bone-like apatite elicit cells with many of the features of osteoclasts.

Tartrate-resistant acid phosphatase (TRAP) activity was localized to multinucleated cells in contact with BPs or particles of Ap/OC. We found that one of five lots of Ap generated a weak TRAP reaction 12 days after implantation. It is possible that the partial reaction may have been due to absorption of endogenous osteocalcin or other proteins following implantation. It is well known that osteocalcin and many serum proteins accumulate in bone matrix because of

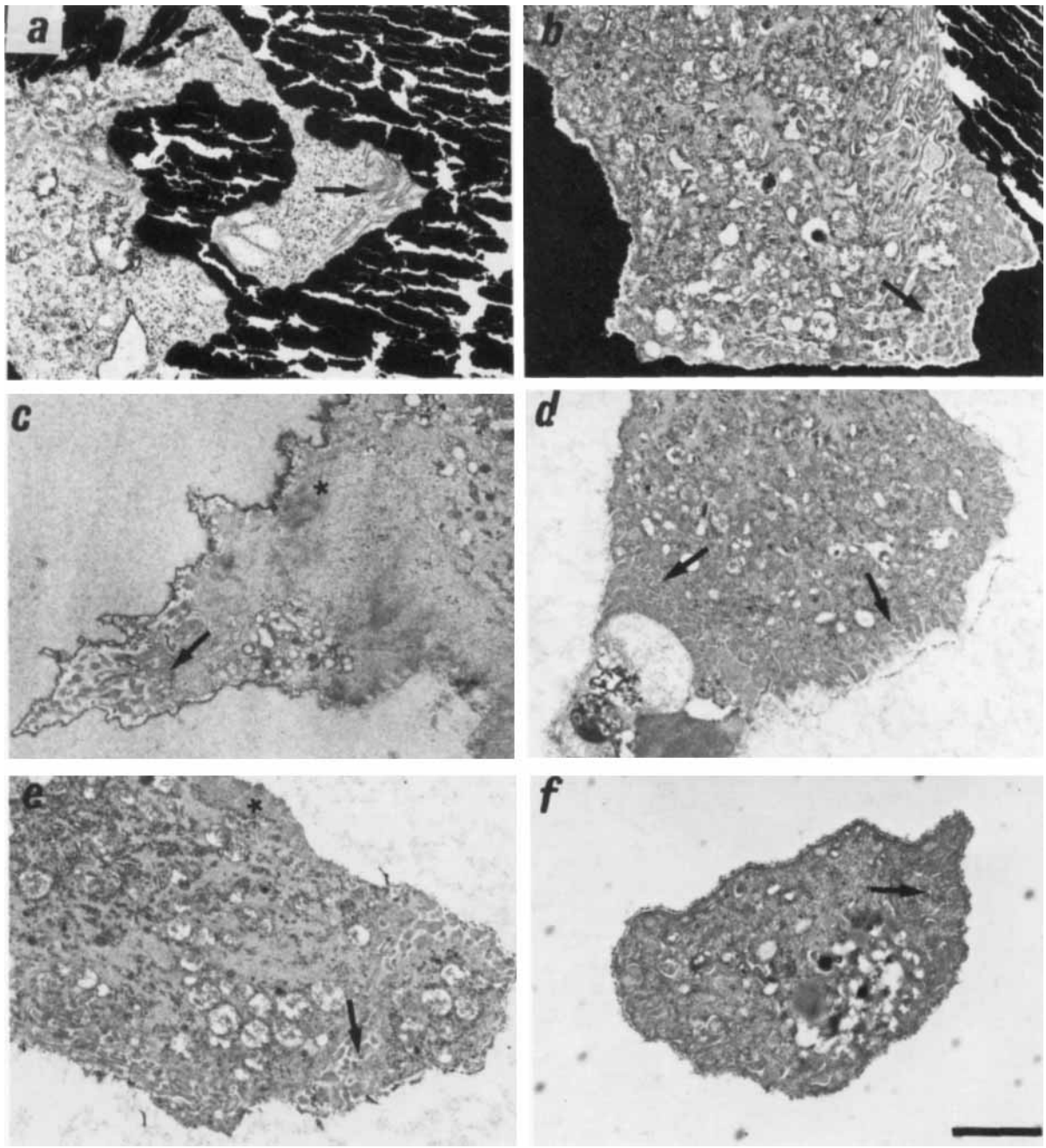

Fig. 7. Transmission electron micrographs of membrane specializations in cells associated with Ap/OC particles. Asterisks indicate clear zones of attachment and arrows show various forms of ruffled borders distinct from convoluted microvilli (M). **a** and **b** were not decalcified; **c–f** were decalcified. ×7,230 magnification.

their affinity for bone mineral [29]. We have previously shown absence of TRAP activity in foreign body multinucleated cells recruited to particulate polymethylmethacrylate and polyethylene [7], two implant materials associated with loosened total hip replacements [31]. Thus, substrate composition influences the phenotype of elicited multinucleated cells. The new microphotometric assay we developed allows objective and quantitative reporting of differences in histochemical reactivity. Some investigators have failed to discriminate, on the basis of TRAP activity or ruffled borders, between foreign body giant cells and cells recruited by other prepara-

tions of bone implants [32,33]. We attributed these differences to differences in the size and composition of the bone preparations, residual inflammatory cellular debris, and the time of sampling [34]. Immunohistochemical reactivity with rabbit IgG against rat bone purple acid phosphatase (which is the tartrate-resistant form of the enzyme) corresponded to the distribution of enzyme activity. This rules out the possibility that the cells refered to as foreign body giant cells possess inactive enzyme.

In agreement with our work in rodents, Krukowski and Kahn described osteoclast-like cells that were formed upon implantation of bone powder onto the chorioallantoic membranes of chick embryos [35]. Those cells were distinguished from multinucleated giant cells that developed around particulate eggshell or commercially obtained hydroxyapatite and from mononuclear fibroblasts or macrophages that surrounded demineralized bone powder. More recently, using an antibody against chicken osteoclasts, Webber et al. showed specific staining of cells associated with commercial hydroxyapatite preabsorbed with bone extracts or osteocalcin [36].

Synthetic Ap/OC particles did not generate as extensive a TRAP reaction as did BPs. It is possible but not yet known whether other matrix proteins influence osteocalcin's enhancement of TRAP activity. It is likely that other bone matrix components contribute to osteoclast differentiation in vivo. Osteocalcin-deficient BPs were able to recruit some osteoclasts and were resorbed to some extent, although far less so than, and delayed in comparison to, control bone. Other matrix signals may have been responsible for the low level of recruitment. For example, collagen peptides and plasma α_2HS glycoprotein [37] are bone-associated molecules that have been shown to be chemotactic for mononuclear cells that may be related to osteoclast precursors. That constituents other than osteocalcin may have some osteoclastogenic activity may explain why major resorptive abnormalities have not been observed in animals treated with warfarin [38,39]. Young rats develop overmineralization and closure of the growth plant when treated with warfarin, a drug which inhibits synthesis of gla residues in vitamin K-dependent proteins [38]. Thus, another protein for consideration is the matrix Gla protein (MGP), found in cartilage and other tissues in addition to bone [40]. In early studies protein compositional changes in bone matrix from warfarin-treated animals (by amino acid analysis and one-dimensional gradient gel electrophoresis) were studied. Proteins in the EDTA extract and guanidine soluble matrix ranging from 100 kD to 24 kD (phosphoproteins, osteonectin, collagen) did not appear to be altered. The only changes noted were the 95–98% decreased osteocalcin levels and a 70–75% reduction in the MGP [41]. Although MGP is present in 6 week-old-rat bone at 1/1,000 the concentration of osteocalcin, its contribution to the observed defect in resorption of BP from warfarin-treated animals cannot be ruled out. Nevertheless, the present data clearly demonstrate that osteocalcin, as the sole component of a synthetic apatite preparation, has the ability to promote differentiation of recruited cells to a multinucleated, TRAP-positive cell with ultrastructural features characteristic of osseous osteoclasts. In contrast, multinucleated giant cells were elicited by apatite particles containing no protein or containing the control proteins, collagen or BSA. The striking differences between the cellular features of tissues elicited by hydroxyapatite implant and Ap/OC particle implant material demonstrate the usefulness of this model to further evaluate the role of potential non-collagenous proteins in promoting resorption of mineralized particles. These data do not reveal the mechanisms whereby osteocalcin or its properties (α-helical structure, gla-residues, or the chemoattractant C-terminal peptide [42]) influence osteoclast differentiation. Further understanding of the contribution of other bone matrix constituents to this process is necessary to define the mechanism of BP resorption.

ACKNOWLEDGMENTS

We are grateful to Pamela Marcom for manuscript preparation. This work was supported by grants from the U.S. Public Health Service to J.G. (AR 31330), to J.L. (AR35166), and to M.J.G. (AR 34078, AR 34081) and by grants to M.J.G. from the Peabody Foundation, Inc., and from the Bristol Myers/Zimmer Corporation.

This work was presented in part at the Second International Workshop on Cells and Cytokines in Bone and Cartilage, Davos, Switzerland, 1988.

REFERENCES

1. Gothlin G, Ericsson JLE: Clin Orthop 120:201–231, 1976.

2. Gay CV: Crit Rev Poultry Biol 1:197–210, 1988.
3. Minkin C: Calcif Tissue Int 34:285–290, 1982.
4. Nicholson GC, Moseley JM, Sexton PM, Mendelsohn PA, Martin TJ: J Clin Invest 78:355, 1986.
5. Mundy GR, Roodman GD: Osteoclast ontogeny and function. In Peck WA (ed): "Bone Min Res." Vol. 5 Amsterdam: Elsevier, 1987, pp 209–279.
6. Glowacki J, Altobelli D, Mulliken JB: Calcif Tissue Int 33:71–76, 1981.
7. Glowacki J, Jasty M, Goldring M: J Bone Min Res 1:327–331, 1986.
8. Glowacki J, Cox KA: Calcif Tissue Int 39:97–103, 1986.
9. Goldring M, Roelke M, Glowacki J: J Bone Min Res 3:117–120, 1988.
10. Glowacki J, Wilcon S, Clark S, Anderson T, Toverud S: J Bone Min Res 3:S98, 1988.
11. Glowacki J: 1984. Morphometric analysis in aging bone. In Gennari C, Segre G. (eds): "Osteoporosis: Social and Clinical Aspects." Amsterdam: Excerpta Medica, 1984, pp 103–108.
12. Glowacki J: Life Sci 33:1019–1024, 1983.
13. Mariano M, Spector WG: J Pathol 113:1–19, 1974.
14. Lian JB, Tassinari M, Glowacki J: J Clin Invest 73:1223–1226, 1984.
15. Glowacki J, Lian J: Cell Differ 21:247–254, 1987.
16. DeFranco D, Glowacki J, Cox KA, Lian J: Calcif Tissue Int, in press.
17. Gundberg CM, Hauschka PV, Lian JB, Gallop PM: Methods Enzymol 107:517–544, 1984.
18. Glowacki J, Mulliken JB: Clin Plast Surg 12:233–241, 1985.
19. Pinta M: 1971. "Spectrometrie d'Absorption Atomique." Paris, Masson, pp 551–553.
20. Charlot G: 1966. "Les Methodes de la Chimie Analytical: Analyse Quantitative Minerale," 5th Edition. Paris: Masson, pp 853–855.
21. Higuchi S, Suga M, Dannenberg AM, Schofield BH: Stain Technol 54:5–12, 1979.
22. Wallenstein S, Zucker CL, Fleiss JL: Circ Res 47:1–9, 1980.
23. Clark SA, Ambrose WW, Anderson TR, Terrell RS, Toverud SU: J Bone Min Res 4:399–405, 1989.
24. Posner AS: Clin Orthop 200:87–99, 1985.
25. Grynpas MD, Tenenbaum HC, Holmyard DP: Connect Tissue Res 21:227–237, 1989.
26. Rey C, Collins B, Goehl T, Dickson IR, Glimcher M: Calcif Tissue Int 45:157–164, 1989.
27. Rey C, Shimizu M, Collins B, Glimcher MJ: Calcif Tissue Int 46:384–394, 1990.
28. LeGeros R, Balmain N, Bonel G: Calcif Tissue Int 41:137–144, 1987.
29. Hauschka PV, Wians FH, Jr: Anat Rec 224:180–188, 1989.
30. Spencer M, Grynpas M: J Chromatogr 166:423–434, 1978.
31. Goldring SR, Schiller AL, Roelke M, Rourke CM, O'Neill DA, Harris WH: J Bone Joint Surg [Am] 65:575–584, 1983.
32. Popoff SN, Marks SC, Jr: Am J Anat 177:491–503, 1986.
33. Walters LM, Schneider GB: Bone Min 4:49–62, 1988.
34. Glowacki J, Cox KA, Wilcon S: Bone Min 5:271–278, 1989.
35. Krukowski M, Kahn AJ: Calcif Tissue Int 34:474–479, 1982.
36. Webber D, Osdoby P, Hauschka P, Krukowski M: J Bone Min Res 5:401–410, 1990.
37. Malone JD, Teitelbaum SL, Griffin GL, Senior RM, Kahn AJ: J Cell Biol 92:227–238, 1982.
38. Price PA, Williamson MK, Haba T, Dell RB, Jee WSS: Proc Natl Acad Sci USA 79:7734–7738, 1982.
39. Feteih R, Tassinari MS, Lian JB: J Bone Min Res 5:885–894, 1990.
40. Fraser JD, Price PA: J Biol Chem 263:11033–11036, 1988.
41. Lian JB, Dunn K, Key LL, Jr: Endocrinology 118:1636–1642, 1986.
42. Mundy GR, Poser JW: Calcif Tissue Int 35:164–168, 1983.