# Recruitment of Osteoclast Precursors by Purified Bone Matrix Constituents

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ABSTRACT The osteoclast, the multinucleated giant cell of bone, is derived from circulating blood cells, most likely monocytes. Evidence has accrued that is consistent with the hypothesis that the recruitment of monocytes for osteoclast development occurs by chemotaxis. In the present study, we have examined the chemotactic response of human peripheral blood monocytes and related polymorphonuclear leucocytes to three constituents of bone matrix: peptides from Type I collagen, $\alpha_2$HS glycoprotein, and osteocalcin (bone *gla* protein). The latter two substances are among the major noncollagenous proteins of bone and are uniquely associated with calcified connective tissue.

In chemotaxis assays using modified Boyden chambers, Type I collagen peptides, $\alpha_2$HS glycoprotein, and osteocalcin evoke a dose-dependent chemotactic response in human monocytes. No chemotaxis is observed in PMNs despite their ontogenetic relationship to monocytes and their documented sensitivity to a broad range of other chemical substances. Our observations are consistent with the view that osteoclast precursors (monocytes) are mobilized by chemotaxis, and suggest that the chemoattractants responsible for this activity are derived from the bone matrix or, in the case of collagen and osteocalcin, directly from the osteoblasts which produce them.

Osteoclasts are multinucleated giant cells that originate through the fusion of uninucleated precursors (1–4). Within the past five years, substantial evidence has accumulated which indicates that these precursors are derived from hematopoietic stem cells located in bone marrow, and that they are carried to sites of incipient or active bone resorption by the blood vascular system (5–7). The available evidence strongly suggests that this precursor cell either is a mononuclear phagocyte (i.e., a macrophage or monocyte) or is derived from the same progenitor which gives rise to mononuclear phagocytes (8–11).

It has been proposed that monocytes are recruited from the circulation to sites of osteoclast differentiation by chemotaxis (12–14). This suggestion follows from the observation that mononuclear phagocytes exhibit directional migration when placed in a gradient of conditioned medium collected from cultures of resorbing bone rudiments (15) and are chemotactically attracted to a partially purified factor isolated from neonatal mouse bone (16). Clearly, both conditioned media and bone extracts are likely to contain a number of factors capable of eliciting a chemotactic response in cells as broadly sensitive to environmental stimulation as mononuclear phagocytes (17), including some which are probably not present under normal conditions of bone turnover. However, the osteoclast is a cell specifically associated with bone and, consequently, it seems reasonable to suppose that, if chemotaxis is involved in mobilizing osteoclast precursors, the chemoattractants originate from the cells or the extracellular matrix which characterize this tissue.

Mature bone matrix is a complex, highly mineralized (~65% dry weight) material built upon a structural framework of Type I collagen (~25–30%) and containing a comparatively small number of noncollagenous proteins and glycoproteins (<5%) (18). Two of the better characterized of the latter proteins are $\alpha_2$HS glycoprotein and osteocalcin. $\alpha_2$HS glycoprotein is synthesized in the liver and is selectively accumulated, relative to other plasma proteins, in bone and other mineralized tissues (19, 20). Osteocalcin (bone *gla* protein) is similarly associated with a range of mineralized tissues but, at least in bone, appears to be synthesized locally, probably by osteoblasts (21–23). Both osteocalcin and $\alpha_2$HS glycoprotein have been implicated in the mineralization process (18, 24).

In the present study, we have attempted to estabish whether $\alpha_2$HS glycoprotein, osteocalcin, and mammalian collagenase-produced fragments of Type I collagen might serve as the chemoattractive agents involved in the recruitment of osteoclast precursors. It has already been shown that peripheral monocytes respond to bacterial enzyme digests of Type I collagen (17) and, in fact, to dipeptides and tripeptides containing glycine and proline, amino acids closely identified with the primary structure of collagen (25). Our data demonstrate that

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human peripheral monocytes respond chemotactically to osteocalcin and $\alpha_2$HS glycoprotein as well as to collagen peptides derived through the action of mammalian collagenase. Polymorphonuclear leucocytes (PMNs) do not exhibit these responses despite their known sensitivity to other chemotactic stimuli (26) and their ontogenetic relationship to monocytes (27–29).

## MATERIALS AND METHODS

### Cell Isolation

Mononuclear cells and PMNs were separated from freshly drawn, heparinized human blood according to the Ficoll-Hypaque density gradient technique (30). The cells were washed in Eagle's minimal essential medium with L-glutamine ($\alpha$-MEM [alpha modification]; KC Biological, Lenexa, Kans.) and were suspended to a final concentration of $2.5 \times 10^6$ mononuclear cells or $1.5 \times 10^6$ PMNs per milliliter in $\alpha$-MEM or in $\alpha$-MEM supplemented with 10% heat-inactivated (60°C for 45 min) fetal calf serum ($\alpha$-10 HI). 0.7 ml of cell suspension was then pipetted into the upper compartment of modified Boyden chambers (AHLCO Mfg. Co., Southington, Conn.).

### Chemotaxis Assay

The chemotactic activities of osteocalcin, $\alpha_2$HS glycoprotein, and Type I collagen peptides were determined by use of the double-membrane technique developed by Campbell (31). The lower membrane was cellulose acetate, pore size 0.45 μm (Millipore Corp., Bedford, Mass.); the upper membrane (facing the cells) was polycarbonate (Nucleopore Corp., Pleasanton, Calif.), pore size either 2 μm for PMN assays or 5 μm for monocytes. The chambers were prepared in triplicate for each variable and were incubated in humidified air at 37°C. After 60 min (for PMNs) or 120 min (for monocytes), the membrane pairs were fixed in isopropanol, stained with hematoxylin, and mounted on slides. The slides were then coded, randomized, and read without reference to the code. Chemotactic activity was quantified on the basis of the number of cells located at the interface between the two membranes and falling within the boundaries of an eyepiece grid at × 400 magnification. Five random grids were read for each membrane pair. The results were expressed as the mean number of cells/grid ± SE, for each set of triplicate samples, and were corrected for random cell migration as determined from scoring control chambers having only medium in the lower compartment. Statistical analysis was made using Student's $t$ test of means.

The positive control in all experiments was $C_5$-derived chemotactic activity ($C_{5a}$), prepared from zymosan-activated human serum (a gift from Dr. D. Kreutzer, University of Connecticut, Farmington, Conn.) and standardized for 50% maximum chemotactic activity ($ED_{50}$) against human monocytes (32, 33). Twice the $ED_{50}$, in 25 μl, was added to the lower compartment of each positive control chamber. Both the test substances and the $C_{5a}$ were diluted to the desired concentration in either $\alpha$-MEM or $\alpha$-10 HI. These media were supplemented with 100,000 U/liter penicillin and 100 mg/liter streptomycin, and were filter-sterilized before use.

### Preparation of Test Materials

Type I collagen peptides (~7,500 mean mol wt) were produced by digesting native guinea pig skin collagen with purified mammalian collagenase and were generously provided by Dr. J. Jeffrey (Washington University School of Medicine, St. Louis, Mo.). Human $\alpha_2$HS glycoprotein (49,000 mol wt) was isolated from human serum using the purification sequence described by Triffitt et al. (20) and was kindly prepared for us by Dr. Triffitt (Medical Research Council Bone Research Laboratory, Oxford, England). Osteocalcin (5,670 mol wt) was extracted from lyophilized chicken bone with EDTA and purified by gel filtration and ion-exchange chromatography according to the methods described by Hauschka and collaborators (21). This substance, which was determined to be free of collagen contamination, was provided by Dr. Hauschka (Harvard University School of Dental Medicine, Cambridge, Mass.).

## RESULTS

Human peripheral monocytes respond chemotactically to collagen peptides produced by the action of mammalian collagenase. The magnitude of the response is dose dependent within the range tested (6.25–100 μg/ml) and is optimal at 12.5–25 μg/ml ($1.7–3.3 \times 10^{-6}$ M) (Fig. 1 *a*). At these latter concentrations, collagen peptides are 40–50% as effective as the positive control substance, $C_{5a}$. Net migration is not observed when the gradient is abolished by placing collagen peptides in both compartments of the Boyden chamber or exclusively in the cell-containing, upper compartment (Fig. 1 *b*). Similarly, no chemotaxis is observed when mammalian collagenase alone is used as a test substance (data not shown).

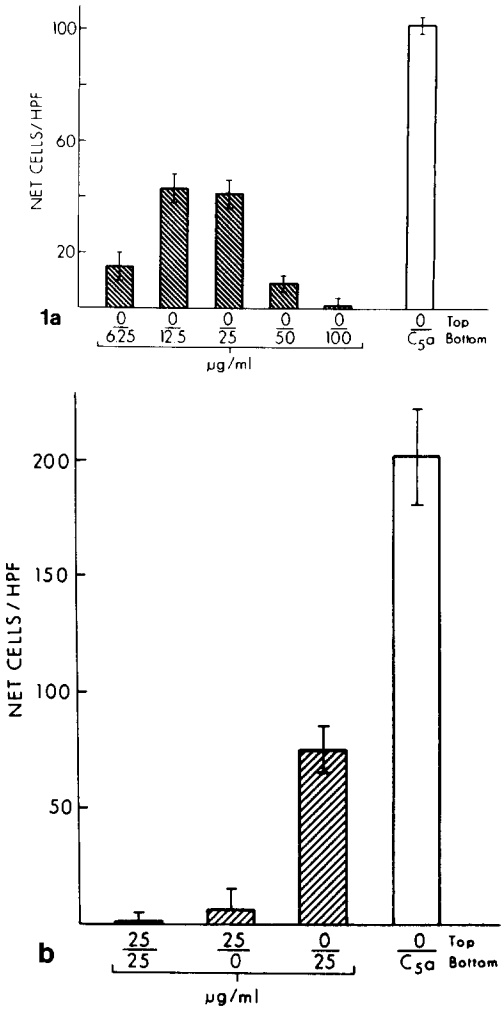


FIGURE 1 (*a* and *b*) The chemotactic response of human peripheral monocytes to peptides from Type 1 collagen. Note that monocytes are optimally responsive to collagen peptides at concentrations of 12.5 and 25 μg/ml ($1.7–3.3 \times 10^{-6}$ M) (*a*) and that the response is abolished in the absence of a gradient (*b*). The "fractions" used in labeling the abscissa indicate the location of the test substance in the Boyden chamber (top [upper] or bottom [lower] compartment) and its concentration in μg/ml. Striped bars denote chambers containing test material; open bars, chambers containing the positive control substance, $C_{5a}$. Values are expressed as net cells/HPF (high-power field) and are plotted ± SEM.

Purified osteocalcin also promotes a dose-dependent chemotactic response in monocytes (Fig. 2 *a*). This response, elicited over the range of 6.25–100 μg/ml, is optimal at 12.5–25 μg/ml ($2.2–4.3 \times 10^{-6}$ M). Chemotaxis fails to occur if the osteocalcin is placed in both compartments of the Boyden chamber or if the protein is present only in the upper compartment (Fig. 2 *b*). (In fact, in the particular experiment illustrated in Fig. 2 *b* the presence of osteocalcin in the upper compartment significantly inhibited ($P < 0.01$) the net migration of cells into the membrane). Neither osteocalcin nor collagen peptides used over the same concentration range evoke a chemotactic response in PMNs (data not shown).

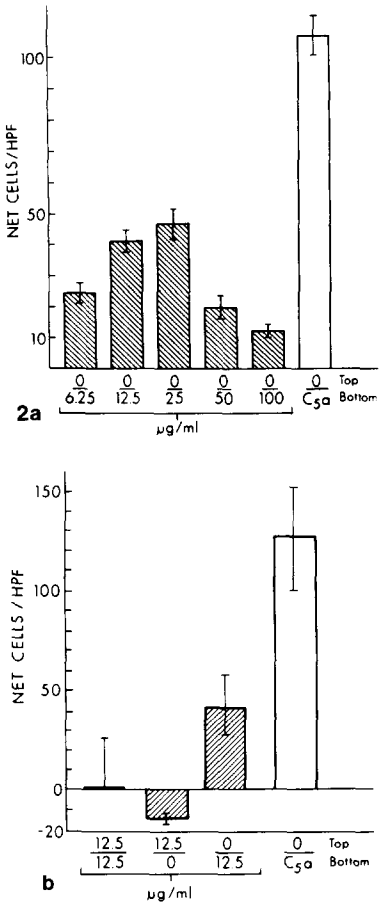


FIGURE 2 (*a* and *b*) The chemotactic response of human peripheral monocytes to osteocalcin. Note that the cells show significant directed migration to osteocalcin at concentrations ranging from 6.25 to 100 μg/ml and that they are optimally responsive at 12.5 and 25 μg/ml ($2.2–4.3 \times 10^{-6}$ M). Note also that a net positive migration is only observed when the chemoattractant is present in the lower (bottom) compartment of the Boyden chamber (*b*).

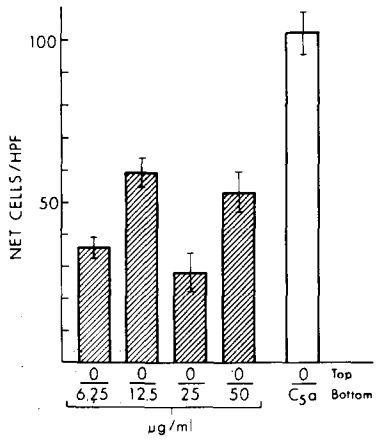


FIGURE 3 The in vitro migration of human peripheral monocytes in a gradient of $\alpha_2$HS glycoprotein. Note that the magnitude of the response to this serum glycoprotein and the effective concentration ($1.3 \times 10^{-7}$ to $1.02 \times 10^{-6}$ M) are similar to those observed with collagen peptides and osteocalcin.

The limited availability of purified $\alpha_2$HS glycoprotein precluded our fully assessing the chemotactic nature of this compound. However, the results, to date, demonstrate that the glycoprotein does elicit the increased migration of monocytes under conditions identical to those employed in collagen peptide and osteocalcin assays, and is effective at comparable concentrations, i.e., between 6.25 and 50 μg/ml ($1.3 \times 10^{-7}$ to $1.02 \times 10^{-6}$ M) (Fig. 3).

## DISCUSSION

Osteoclast development occurs under physiological conditions at sites within the skeleton which are undergoing (or about to undergo) bone resorption. The first step in this process, the mobilization of precursor cells, appears to result from the chemotaxis of mononuclear phagocytes (15, 16), cells known to exhibit directional migration in response to a variety of chemical agents (17). Since osteoclast development is a tissue-specific phenomenon and is generally linked to other events associated with normal bone turnover, it follows that the chemotactic factor (or factors) responsible for mobilizing the precursor cells should exhibit a similar tissue specificity and become available as a consequence of the processes of bone formation or resorption.

Because the organic constituents of bone matrix largely define the microenvironment in which osteoclasts are formed, these constituents would clearly fulfill the "geographic" requirement for chemoattractants which might be involved in the recruitment of osteoclast precursor cells. In addition, such substances would become available as recruiting agents when bone matrix is broken down during osteolysis or, in the case of matrical components produced *in situ*, as a direct result of secretion by osteoblasts lining the bone surface. In fact, as documented below, the bone literature contains reports consistent with both modes of chemoattractant release.

When bone is stimulated to resorb, by any of a number of chemical agents, the osteoclast population characteristically responds with a burst of resorptive activity by extant cells followed, after a lag period of several hours, by the generation of new, and often larger, osteoclasts (34–37). During this lag period, the mineral component of the bone matrix is mobilized and, along with it, $\alpha_2$HS glycoprotein, osteocalcin, and, probably, collagen. These substances would then serve to attract monocytes (precursor cells) to sites of osteoclast differentiation. In support of this possible mechanism of precursor recruitment, we note that demineralization is the first phase of bone resorption (38), and that both $\alpha_2$HS glycoprotein and osteocalcin are released from bone when the mineral component of the matrix is removed (19, 24). We also note that urinary excretion of hydroxyproline (presumably derived from the degradation of bone collagen) is elevated during periods of bone resorption (39) and that plasma levels of osteocalcin (bone *gla* protein) are increased in bone disease characterized by high bone turnover (40).

There are other circumstances, such as those encountered in embryonic and fetal life, where osteoclasts appear *de novo*, in the absence of any apparent, prior resorptive activity. Here, it seems reasonable to speculate that the secretion of soluble chemoattractive matrix constituents by osteoblasts is responsible for attracting osteoclast precursor cells. In support of this proposition, we note that osteoblast-derived osteogenic sarcoma cells synthesize and release osteocalcin (bone *gla* protein) in vitro (41), and that the level of synthesis of this compound can be significantly increased by exposing the cells to parathyroid hormone (PTH) (42). In addition, the serum level of bone *gla* protein is increased in rats after the injection of $1,25(OH)_2D_3$ (43). Finally, we call attention to the growing

body of evidence indicating that PTH acts to enhanced resorption in vitro via receptors located on osteoblasts rather than osteoclasts (44).

The results of the present study demonstrate that monocytes respond chemotactically to osteocalcin, to $\alpha_2$HS glycoprotein, and to Type I collagen peptides produced through the action of a purified mammalian collagenase. By contrast, PMNs do not respond to either osteocalcin or collagen peptides despite the fact that they are derived from the same hematopoietic stem cell as monocytes (27–29) and are chemotactically sensitive to a broad spectrum of other substances (25). These findings support the hypothesis that chemotaxis plays a major role in the recruitment of osteoclast precursor cells, and suggest that the likely sources for the relevant chemoattractive agents are the bone matrix and the osteoblast.

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