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Identification of a Bone Matrix-derived Chemotactic Factor

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Summary. When demineralized bone matrix powder is implanted subcutaneously in the rat, the early responses involve the appearance and proliferation of mesenchymal cells at the site of implantation, followed by cartilage and bone formation. The ability of cells to migrate to the implant suggests that chemotaxis may be a critical event in this process. Therefore, using the modified Boyden chamber assay, we tested extracts of demineralized bone matrix for chemotactic activity. We have identified and partially purified, on molecular sieve chromatography, a heat labile and trypsin-sensitive protein (M_r = 60,000–70,000) that is a potent chemoattractant for mouse calvaria, osteoblast-like cells (MMB-1), but not for monocytes (putative osteoclast precursors). These findings suggest that chemotactic protein(s) have a significant role in the recruitment of osteoprogenitor cells to a site of bone repair.

Key words: Chemotaxis — Bone — Osteoblasts — Bone proteins.

Chemotaxis, the directed migration of cells in response to a chemical gradient, is thought to play a major role in wound healing. During this process, neutrophils, macrophages, and mesenchymal cells enter the site of injury to remove debris or mediate tissue repair [1]. Although the precise mechanisms involved are poorly understood, this movement of cells into the wound site is thought to involve their directed migration in response to specific signals. For example, factors such as fibronectin and platelet-derived growth factor (PDGF) are known to be chemoattractants for fibroblasts [2, 3], as well as for other cells [4, 5] involved in wound healing. Since the early stages of wound and fracture healing are similar in that exogenous cells migrate to the site of repair, it would appear that chemoattractants are involved in both these processes. Interestingly, fibronectin has been found on the surface of osteoblasts during bone formation [6]. Also, a factor with characteristics similar to PDGF has been isolated from the media of cultured osteosarcoma cells [7], but it is not known whether this factor can stimulate cell migration. Further, it has been reported [8, 9] that other bone constituents can induce chemotaxis in monocytes. Since monocytes have been postulated to be osteoclast precursors, chemotaxis might well be involved in the migration of both osteoprogenitor and resorptive cells to areas of bone induction.

We have tested extracts of demineralized bone powder for chemotactic activity. These bone powders contain all the information necessary to induce new bone formation when implanted subcutaneously. The new bone is formed in a manner analogous to fracture healing [10]. During this ectopic bone formation, a regulated sequence of events occurs. Initially, mesenchymal cells enter the implantation site; subsequently, new cartilaginous tissue is formed and this is replaced by bone; finally, marrow forms within the bony ossicle. Thus, chemoattractants would appear to have some function in bringing cells to the site of implantation. In this study, we identified a bone-derived cheomtactic protein and have partially characterized it. This protein was obtained from 4M guanidine HCl extracts of demineralized bone matrix powder and elicits a chemotactic response from mouse calvaria cells, but not from monocytes.

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Materials and Methods

Cell Culture

Two types of cells were examined: osteoblast-like bone cells and human mononuclear cells. Osteoblast-like bone cells were a gift from Dr. Richard Luben (University of California, Riverside). These cells (MMB-1 cloned cells from mouse calvaria) are reported to exhibit several characteristics of osteoblasts, such as PTH-induced stimulation of adenyl cyclase and decreased collagen synthesis in response to PTH [11]. The cells were cultured in a 1:1 ratio of Ham's Modified F12 and Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Reheis Chemical Corporation) and gentamicin (50 μg/ml). Human mononuclear cells, a gift from Dr. Douglas Strong, Naval Medical Research Institute, Bethesda, Maryland, containing approximately 15% monocytes, were stored frozen in liquid nitrogen and thawed at 37°C immediately prior to use, as reported elsewhere [12].

Coating of Filters

In chemotactic assays involving bone cells, Nucleopore filters (8 μm pore membranes, Nucleopore Corporation) were first coated with a gelatin solution (Sigma Chemical Co., 20 μg/ml), as described previously [13]. For studies on monocyte chemotaxis, we found that 5 μm uncoated, detergent-free filters gave the most reproducible results.

Chemotactic Assay

The chemotactic assays were performed in modified Boyden chambers according to the method of Postlethwaite et al. [13]. The frozen monocytes were thawed quickly at 37°C, rinsed once in Gey's balanced salt solution (BSS) containing 2% bovine serum albumin (BSA, Miles Laboratories, Inc.), and diluted to a final concentration of $4-5 \times 10^6$ cells/ml. The osteoblast cultured cells were detached from dishes with 0.08% trypsin/0.04% ethylenediamine-tetraacetic acid (EDTA) and rinsed once with DMEM and soybean trypsin inhibitor (Worthington, 1 mg inhibitor/0.99 mg trypsin). These cells were then resuspended in DMEM, containing 1 mg/ml BSA, to a final concentration of $3-4 \times 10^5$ cells/ml. Test samples were diluted in either Gey's BSS with 2% BSA (for monocytes) or DMEM with 1% BSA (for the bone cells); 30 μl of this attractant-containing solution was added to the lower well of a Boyden chamber. A polycarbonate filter was then placed over this well, and 0.2 ml of the cell suspension was added to the upper well.

The chambers were incubated at 37°C in a 5% CO_2/95% air mixture for 4 h for the bone cells and 2 h for the monocytes. The filters were then removed, stained with Diff-Quik (Harleco) and transferred to slides. The cells attached to the upper surface of the filter were removed with a cotton swab and the chemotactic response was determined by counting the number of cells migrating to the lower surface of the filter in 1 mm^2 microscopic fields (33 fields at 430×). Samples were assayed in duplicate for each experiment, and the final chemotactic activity was expressed as the mean obtained from the data of pooled experiments. The standard error of the mean did not exceed 15%. Unless otherwise specified, all assays were performed on the unfractionated, ammonium sulfate-precipitated extract from the bone matrix (see below).

Isolation of the Bone Chemotactic Factor

The demineralized bone matrix powder was prepared from the tibia and femurs of adult Long-Evans and Sprague-Dawley rats, as described previously [10]. Bone matrix factor was extracted with 0.05 M Tris HCl buffer (pH 7.4, 20 ml/g powder) containing 4 M guanidine HCl and protease inhibitors (0.05 M 6-aminohexanoic acid, 0.005 M benzamidine HCl, 0.001 M phenylmethylsulfonyl fluoride) at 4°C, over a 3-day period. The extract was dialyzed against distilled water. All of the chemotactic activity precipitated with ammonium sulfate at 50% saturation. The precipitate was dissolved in distilled water, dialyzed against water, and lyophilized. The chemotactic activity in this precipitate was partially purified by gel filtration chromatography on Sepharose CL-6B (Pharmacia) columns equilibrated in 4 M guanidine HCl exactly as described previously for the separation of other bone matrix proteins [14]. Five ml fractions were collected; aliquots (0.2 ml) were dialyzed against DMEM, and then assayed for chemotactic activity. Aliquots of each fraction were also analyzed, following reduction, by electrophoresis on 10% SDS-polyacrylamide gels, according to the method of Laemmli [15]. Molecular weight standards were obtained from Bio-Rad.

Heat Stability and Trypsin Sensitivity of the Bone Chemotactic Activity

For heat stability studies, 500 μg/ml of the ammonium sulfate precipitated material was incubated in DMEM with 1% BSA for 30 min at various temperatures and then assayed for chemotactic activity. Protease sensitivity was assessed by incubating 500 μg/ml of this material in the presence of trypsin (1 mg/ml) for 30 min at 37°C. Soybean trypsin inhibitor (1 mg inhibitor/0.99 mg trypsin) was added before assaying for chemotactic activity. Two controls were performed in the protease sensitive experiments: (1) DMEM containing 1% BSA was incubated with trypsin for 30 min and then soybean trypsin inhibitor was added prior to the assay, and (2) bone extract (500 μg/ml) containing soybean trypsin inhibitor was assayed directly for chemotactic activity. The expected chemotactic response was not changed in either control.

Results

Chemotactic Properties of Demineralized Bone Matrix Extract

Initially, the demineralized bone powder was extracted sequentially using phosphate buffered saline, 6 M urea and then 4 M guanidine HCl. The chemotactic activity was only present in the guanidine extract. In addition, 4 M guanidine extracts of undemineralized bone powder had no chemotactic activity. Mouse calvaria cells were found to migrate in a concentration-dependent manner towards the guanidine extract of the bone

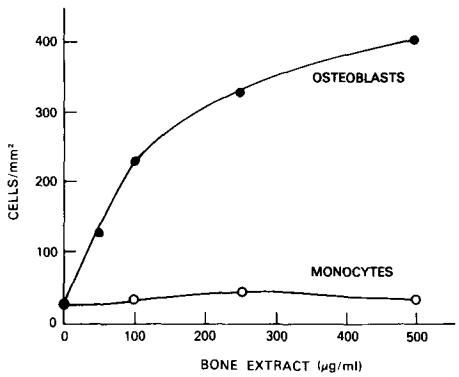

Fig. 1. The response of monocytes and osteoblasts to the bone extract. Gradient (●——●) denotes osteoblast response and (○——○) denotes monocyte response to increasing concentration of attractant in the lower compartment of the Boyden chamber. f-Met-Leu-Phe was a potent chemoattractant for monocytes (350 cell/mm² at 10^{-8}M). The values are taken from the average of 2 experiments. The SEMs did not exceed 11%.

matrix (Fig. 1). In contrast, monocytes were not attracted to the bone matrix extract even at the higher concentrations (Fig. 1). To determine if the bone-derived material stimulated directed (chemotactic) migration of cells, a "checkerboard analysis" was performed according to the method of Zigmond and Hirsch [16] (Table 1). For these assays, various concentrations of the bone extract were added either to the lower compartment, to the upper compartment (with the cells), or to both compartments, thereby establishing a positive, negative, or no gradient, respectively. The cells migrated better in positive gradients than they did in a negative gradient or with no gradient. For example, at a concentration of 250 μg/ml of extract in the lower chamber and no attractant in the upper chamber, the response was about 7-fold greater than that which occurred when the concentrations were reversed (negative gradient), and about 4-fold greater than when there were equal concentrations (250 μg/ml) (no gradient) in both chambers. These data confirm that the bone-derived material is a true chemoattractant.

We next investigated if the chemotactic activity was sensitive to either heat or proteolytic cleavage (Table 2). Almost 50% of the chemotactic activity was lost at 55°C, and at 100°C, the material was essentially inactivated. These data, along with the molecular size estimations described below, suggest that the bone chemotactic activity is not related to PDGF, which is a smaller protein stable to 100°C [17, 18]. At 37°C, the addition of trypsin to the bone extract inhibited chemotactic activity by 90%. These data suggest that the chemoattractant from the bone extract is a protein or has a protein moiety necessary for its activity.

Table 1. Checkerboard analysis of bone chemotactic activity

		Bone extract concentration below filter (μg/ml)			
		0	50	100	250
Bone extract concentration above filter (μg/ml)	0	27	128	231	329
	50	18	25	184	179
	100	14	56	87	50
	250	45	44	102	82

Results are averages of duplicate values whose SEMs did not exceed 11%

Table 2. Effect of temperature on the chemotactic activity

Temperature (°C)	% inactivation
37	0
45	0
55	45
65	75
80	80
100	85

The SEMs did not exceed 15%

Since fibronectin is present in bone [6] and has been shown to be a potent chemoattractant for various cells, including osteoblasts [19], it was important to determine if the chemoattractant activity was due to fibronectin or a fibronectin fragment that may have been present in the bone extract. This was done in 2 ways. First, experiments were performed in a fashion similar to the "checkerboard" analysis described above. If the chemotactic activity of the bone extract in these experiments was due mainly to fibronectin, then addition of fibronectin to the upper well would inhibit net migration of cells towards the bone-derived material in the lower well. The addition of fibronectin to the upper well in an amount found to cause maximal chemotaxis with osteoblasts, (20 μg/ml—data not shown), did not decrease migration of osteoblasts toward bone extract (500 μg/ml) in the lower well. Second, we compared the chemotactic activity and the amount of fibronectin present in extracts of human bone, using the enzyme-linked immunoabsorbant assay (ELISA) [20]. (Guanidine EDTA extracts of embryonic human long bones and rat calvaria also had chemotactic activity [9]). The human bone extract contained only 0.5% fibronectin by weight. Since 5-20 μg of fibronectin is required for a significant amount of migration of osteoblasts (data not shown), it is unlikely that the 2.5 μg, present in our test sample at maximum concentration (500 μg/ml), was responsible for the chemotactic activity. Further, following gel filtration chromatography (see below), fibronectin was not detectable by

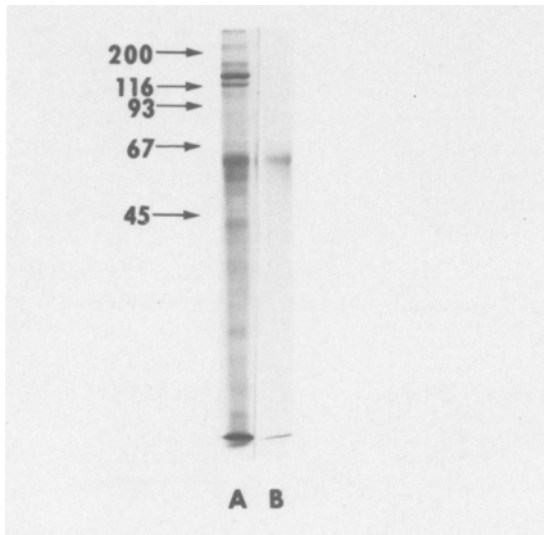

Fig. 2. Ten percent SDS-polyacrylamide gel electrophoresis (reduced): Lane A. Crude bone extract (prior to chromatography). Lane B. Fraction 103 from molecular sieve column (the fraction with the maximum chemotactic activity). Arrows = molecular weight standards.

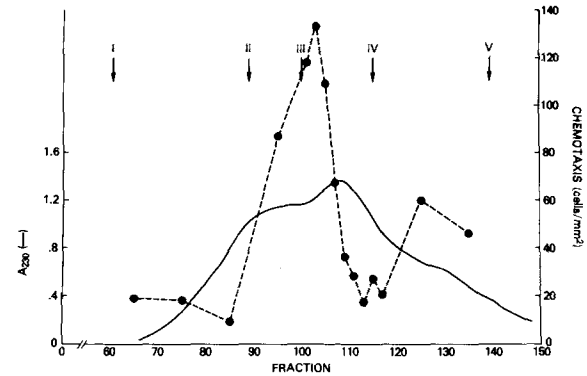

Fig. 3. The bone extract was fractioned using Sepharose CL-6B molecular sieve chromatography. The column was eluted with 4 M guanidine HCl, and 5 ml fractions were collected. Chemotactic assays were performed on the fractions after dialyzing against DMEM. The chemotactic values are averages of 2 experiments. The SEMs did not exceed 10%. **Arrows:** I = blue dextran (2×10^6); II = collagen chains (110,000); III = BSA (67,000); IV = osteonectin (32,000); V = osteocalcin (5,800). Bone extract activity (500 μg/ml, prior to molecular sieve) = 140 cells/mm^2; control (background migration) = 15 cells/mm^2; fraction 103 (100 μg/ml) = 135 cells/mm^2.

ELISA in the human bone fraction having chemotactic activity. These data strongly indicate that the bone chemotactic activity measured in this study is not due to fibronectin.

Partial Purification and Characterization of the Bone Factor with Chemotactic Activity

Gel electrophoresis of the crude material (Fig. 2, Lane A) indicated several high molecular weight components (M_r = 116,000–200,000) and also a number of components between 45,000 and 65,000 daltons. This material was chromatographed on Sepharose CL-6B columns and the fractions assayed for osteoblastic chemotactic activity.

The fractions with the predominant chemotactic activity for the MMB-1 cells were located in the region of M_r = 60,000–70,000 (Fig. 3). On gel electrophoresis, this fraction gave several bands in the region around M_r = 65,000 (Fig. 2, Lane B).

Discussion

We have demonstrated that a component of demineralized bone matrix has chemotactic activity for osteoblast-like cells but not for monocytes. Preliminary data suggest that other connective tissue cells (e.g., human embryonic fibroblasts) also respond to this chemotactic factor. The factor was heat-labile and trypsin-sensitive and therefore is a protein-like material. Partial purification of this factor by molecular sieve chromatography gave a pattern of activity consistent with a 60,000–70,000 dalton protein. This chemoattractant was contained within the mineralized bone matrix space, since the matrix must be demineralized before the chemotactic activity can be solubilized. A similar chemotactic activity was also observed in both embryonic human long bones and rat calvaria [19]. However, other noncollagenous proteins found in bone, including osteocalcin [22, 31], osteonectin [23], and the α-2HS-glycoprotein [24] were not chemoattractants for MMB-1 osteoblasts [19]. Therefore, the bone chemotactic factor does not appear to be related to these bone constituents or to circulating factors, such as fibronectin or PDGF.

These proteins may prove necessary for other activities of bone cells. Osteonectin, for example, binds selectively to collagen and apatite and has been localized to mineralizing bone trabeculae. Osteonectin is able to induce mineral deposition onto type I collagen *in vitro* and thus may be required for the initiation of mineralization *in vivo* [23]. Although major tissue functions for osteocalcin and the α-2HS-glycoprotein have yet to be identified for bone, there is some evidence that these constituents [8, 9] may stimulate potential bone resorptive cells to migrate to areas of bone remodeling. The bone chemotactic factor of our study may be required to induce osteogenic cells to migrate to bone accretion sites. Once such cells reach sites of bone formation, they may then be able to initiate the synthesis of bone-specific proteins, such as os-

teonectin, that regulate osteogenic processes like bone mineralization.

The precise nature and function of all the bone chemotactic factors is uncertain. However, constituents of the bone matrix may be needed to mobilize a variety of cells prior to new bone formation in situations where rapid osteogenesis is required. In support of this hypothesis is the observation that in the *in vivo* bone induction system initial events include the migration of a number of cell types to the site of implantation [10]. Thus, the protein described above may have a primary function in bone development, growth, and remodeling. This factor, therefore, may be useful for stimulating new bone formation in the treatment of nonunion bone fractures and degenerative bone diseases (e.g., periodontal disease).

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