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Osteopontin–a possible anchor of osteoclasts to bone

(bone matrix proteins/cell adhesion/vitronectin receptor)

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ABSTRACT **A key event in bone resorption is the binding of osteoclasts to the mineral matrix of bone surfaces. A candidate for mediating this binding is osteopontin, a major cell- and hydroxyapatite-binding protein synthesized by osteoblasts. In support of this hypothesis is the fact that the synthesis of osteopontin is stimulated by calcitriol (1,25-dihydroxyvitamin(D_3), a substance that induces bone resorption. The present study demonstrates that osteopontin is highly enriched at regions of the bone surface where osteoclasts are anchored. Furthermore, the vitronectin receptor, which has known specificity for osteopontin, is shown preferentially localized at the corresponding area of the osteoclast plasma membrane. The results thus support the hypothesis that osteoclasts when resorbing bone are anchored by osteopontin bound both to the mineral of bone matrix and to a vitronectin receptor on the osteoclast plasma membrane.**

More than 100 years have passed since Kölliker gave the name "Osteoklast" to a large multinucleated cell observed along bone surfaces and suggested a role for the cell in bone resorption (1). It is now well established that osteoclasts are derived from a bone-marrow cell reaching the bone surfaces by means of blood-borne mononuclear precursor cells (2). The stimulus is provided by other bone cells, which when appropriately stimulated, produce factors that induce recruitment of osteoclast progenitor cells to select bone surfaces (3). Osteoclasts are in contact with mineralized bone matrix at two modified cell surface areas, a clear zone and a ruffled border (4, 5) (Fig. 1). Active bone resorption is confined to the ruffled border area, where an acidic milieu is maintained. The clear zone is located immediately adjacent to the ruffled border and is thought to provide the osteoclast with a tight attachment to bone, thereby sealing off the ruffled border zone (5, 6). The mechanism for this vital attachment has remained an enigma. It has, however, been suggested that a vitronectin receptor identified on isolated osteoclasts (7) may be involved (8).

Over recent years several noncollagenous matrix proteins of bone have been isolated and characterized. At least two of these proteins—i.e., osteopontin and bone sialoprotein—are acidic and bind tightly to hydroxylapatite (9, 10). A functional Arg-Gly-Asp (RGD) cell-binding sequence has been identified in both proteins by cDNA cloning and sequencing (11, 12). A receptor for the proteins on cultured osteoblastic cells appears to be the vitronectin receptor (13). Both osteopontin and bone sialoproteins are products of osteoblasts, contain stretches of acidic amino acids, and are phosphorylated. Osteopontin and bone sialoprotein represent distinct gene products. The synthesis of only one of the proteins, osteopontin, is stimulated by calcitriol (14, 15), which is known to induce bone resorption. Thus, osteopontin is a candidate for mediating binding of osteoclasts to bone at the clear zone. In the present study this hypothesis was tested by using ultrastructural immunocytochemistry.

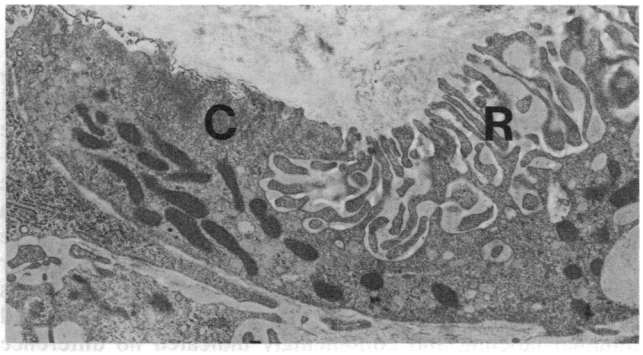

FIG. 1. Low-power electron micrograph of an active osteoclast in the area of eruption of the first molar. R, ruffled border—i.e., area of bone resorption; C, clear zone—i.e., area of bone attachment. (×8100.)

MATERIALS AND METHODS

Six-day-old rats were anesthetized with a single i.p. injection of 0.15 ml of fentanyl/fluanison and perfused for 5 min at room temperature through the left cardiac ventricle with a fixative of 0.1 M phosphate, pH 7.2/0.3% glutaraldehyde/0.3% paraformaldehyde/2% (wt/vol) Dextran T-40 (Pharmacia). The maxillae were dissected out and further fixed by immersion in the same fixative for 1 hr. The specimens were dehydrated and embedded at low temperature in the polar resin Lowicryl K11M (Chemische Werke Lowi GmbH, F.R.G.). Ultrathin sections were cut and placed on carbon-reinforced, Formvar-coated nickel grids. After preincubation with 10% bovine serum albumin/0.01 M phosphate/0.15 M sodium chloride, pH 7.4, consecutive sections were incubated with polyclonal antibodies raised in rabbits against rat osteopontin fusion protein produced in *Escherichia coli* and human vitronectin receptor (a gift from E. Ruoslahti). A negative control was obtained by incubating osteopontin with antiosteopontin overnight before application to the sections. For vitronectin receptor, normal rabbit serum was used as the negative control. Bound antibodies were detected with 10-nm gold-conjugated protein A (Janssen Life Science Products, Belgium) on sections contrasted with uranyl acetate and lead citrate. Electron micrographs were taken along the surface of the bone trabeculae by systematic random sampling (cf. ref. 16). Semiquantitative estimation of immunolabeling was performed by counting gold particles over the different compartments. Reference areas were measured by point counting, and plasma membrane length was measured by intersection counting (16); this procedure allows computation of particle number per unit area and plasma membrane length, respectively (16). Calculations were performed on

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data from at least three blocks from three animals. Micrographs were made from one section per block for each immunostaining—i.e., for osteopontin and for vitronectin receptor. From each section, a minimum of three osteoblasts and three osteoclasts and their adjacent bone matrix, which is the nearest 1 μm of the bone matrix facing the cell membrane, were covered. Thus the data on osteopontin and vitronectin receptor distributions were based on a total of 374 micrographs (primary magnification × 17,000). To allow comparisons, regardless of variability in total number of gold particles between sections, the semiquantitative data were calculated as percent labeling in each compartment.

RESULTS

Active osteoclasts are quite numerous in apposition to bone close to the point of eruption of the first molar (Fig. 1). Labeling of osteopontin was unevenly distributed along the bone trabeculae: High concentrations were seen at the surface of bone facing the clear zone of the osteoclasts, while concentrations were low in bone of the ruffled border area (Table 1, Fig. 2). Furthermore, concentrations of label were low in the bone matrix further away from the surface. Lower degree of labeling was also seen in areas facing osteoblasts (Table 1) and intracellularly in osteoblasts. Controls showed minimal labeling and convincingly indicated no difference between compartments.

The highest concentration of marker for the vitronectin receptor was found at the clear zone area of the plasma membrane of the osteoclast (Table 1). The concentrations of receptor in the ruffled border regions of the plasma membrane were low, as were the concentrations in regions not facing the bone surface (Table 1). Some immunolabeling of receptor was found intracellularly, although without any prevailing distribution in terms of organelles. Controls showed minimal unspecific labeling.

DISCUSSION

After isolation and characterization of osteopontin (9, 10), cloning and sequencing showed that the molecule could bind integrins on cells (11, 13). Studies of the specificity indicated that the vitronectin receptor was a probable candidate for the binding (13). No apparent structural homology can, however, be identified with regard to the primary sequences of osteopontin (11) and vitronectin (17). Their functions and localizations also differ in that vitronectin is not a prominent component of bone (8) but a serum protein attributed a role as a multifunctional regulator in the hemostatic and immune systems (18). Although little is known about the function of osteopontin, the stimulated synthesis by calcitriol (14, 15) may be taken to indicate a role in bone breakdown. Substantiated by semiquantitative data, the present study demonstrates that osteopontin has a preferential localization to the area of bone facing the osteoclast, particularly where the osteoclasts attach—i.e., the clear zone. The specificity of this restricted distribution is supported by data from control experiments and, furthermore, by the fact that the other cell-binding bone protein, bone sialoprotein, is not found close to the osteoclast (unpublished work).

A strikingly similar pattern of immunolabeling between tissue constituents was found in three consecutive incuba-

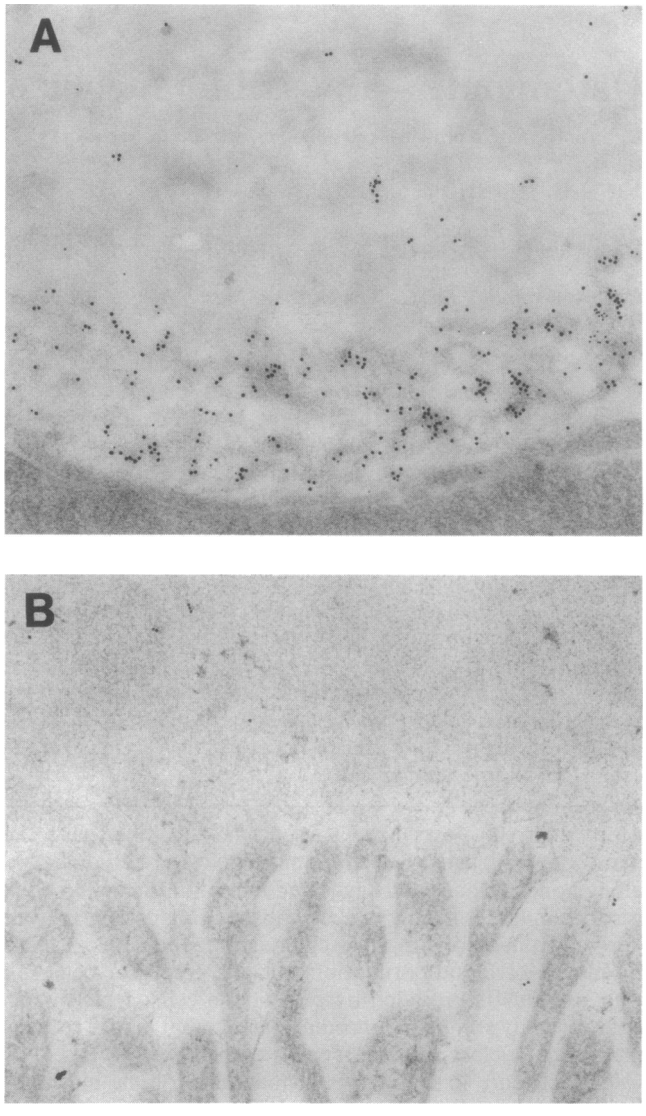

FIG. 2. Immunolocalization of osteopontin in bone facing the osteoclast. (*A*) Most gold particles are seen in the bone matrix close to the cell surface of the clear zone. (*B*) Much lower concentrations of gold particles are seen in the bone matrix of the ruffled border area and deeper in the bone. (×20,800.)

Table 1. Immunoreactivity for osteopontin and vitronectin receptor in bone

	Immunoreactivity, % labeling (mean gold immunolabeling × μm^{-2})*				
	Clear zone	Ruffled border	Osteoblast	Central bone	Vascular septum
Osteopontin	58 ± 7 (79.5)	3 ± 1 (3.0)	33 ± 7 (34.0)	6 ± 2 (11.9)	1 ± 1 (1.0)
Control	14 (0.5)	20 (0.7)	34 (1.2)	20 (0.7)	11 (0.4)
Vitronectin receptor	68 ± 15 (0.64)	9 ± 2 (0.08)	2 ± 1 (0.02)	0 (0)	21 ± 10 (0.20)
Control	22 (0.02)	44 (0.04)	11 (0.01)	0 (0)	22 (0.02)

For osteopontin and the corresponding control, the compartments (clear zone, ruffled border, osteoblast) refer to the nearest 1 μm of the bone matrix facing the cell. For vitronectin receptor and the corresponding control, the compartments refer to cell plasma membrane. Vascular septum (vitronectin receptor) refers to the part of the osteoclast plasma membrane not facing bone matrix.

*Data for osteopontin and vitronectin receptor include % labeling ± SEM. Units for the vitronectin receptor are % labeling ± SEM (mean gold immunolabeling × μm^{-1}).

tions. Due to relatively large variation in the total number of gold particles per section between different incubations, our data are presented as percent label in each of the five compartments of interest, and we refrain from statistical analysis of the semiquantitative data.

Our results show that osteoclasts *in vivo* express a vitronectin class of receptors. In support, Horton (7) has demonstrated that isolated osteoclasts contain a receptor of this type. In previous experiments it has been shown that the osteoblastic ROS 17/2.8 cells also bind both osteopontin and bone sialoprotein *in vitro* by a mechanism apparently involving a vitronectin receptor (13). It may, therefore, seem puzzling that no staining for vitronectin receptor was seen on osteoblasts in the sections (Table 1). Possibly, however, osteoblasts in the tissue do not express this receptor or antibody binding is blocked. Our observation is supported by Davies *et al.* (8) who recently reported absence of vitronectin receptors on osteoblasts *in vivo*.

The present study shows a striking colocalization of osteopontin and vitronectin receptor restricted to the clear zone of osteoclasts. The observation is highly indicative of a key role for osteopontin in anchoring the osteoblast to the bone surface. In this sense, our results seem to represent a critical piece of evidence down a long line of progression in understanding the role of osteopontin and vitronectin receptors in bone biology. Major contributions to this progress are the reports on cDNA cloning and sequencing of osteopontin that include demonstration of the cell-binding sequence (11) and the affinity for hydroxylapatite (9), the production of the protein by osteoblasts (11, 19), the stimulatory effect of calcitriol on the production of osteopontin by osteoblastic cells (14, 15), the observation of osteopontin in bone anlagen just before osteoclast invasion (20), the demonstration of vitronectin receptors on osteoclasts (7), and the observation that antibodies to the vitronectin receptor inhibit osteoclast motility and bone resorption (21).

This study shows that osteopontin is a natural ligand for the vitronectin receptor in bone and suggests that calcitriol-mediated bone resorption is exerted by inducing osteopontin production. The newly produced protein accumulates along the bone surface and attracts and binds osteoclasts and/or corresponding precursor cells, which then form a tight attachment at their clear zones, allowing local bone resorption.

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