Vitronectin Receptor Has a Role in Bone Resorption but Does Not Mediate Tight Sealing Zone Attachment of Osteoclasts to the Bone Surface

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Abstract. During bone resorption, osteoclasts form a tight attachment, the sealing zone, around resorption lacunae. Vitronectin receptor has previously been shown to be expressed in osteoclasts and it has been suggested that it mediates the tight attachment at the sealing zone. In this study we have shown that glycine-arginine-glycine-aspartic acid-serine pentapeptide inhibits bone resorption by isolated osteoclasts and drastically changes the morphology of the osteoclasts. When the vitronectin receptor was localized by immunofluorescence in rat and chicken osteoclasts cultured on bone slices, it was found to be distributed throughout the osteoclast cell membrane except in the sealing zone areas. Immunoperoxidase staining of rat bone sections at the light microscopical level also revealed intense staining of the cell membrane with occasional small unstained areas, probably corresponding to the sealing zones. Immunoelectron microscopy confirmed the results obtained by light microscopy showing specific labeling only at the ruffled borders and basolateral membranes (0.82 and 2.43 gold particles/μm of membrane, respectively), but not at the sealing zone areas (0.06 gold particles/μm of membrane). Both α_v and β_3 subunits of the vitronectin receptor were similarly localized. These results strongly suggest that, although the vitronectin receptor is important in the function of osteoclasts, it is not mediating the final sealing zone attachment of the osteoclasts to the mineralized bone surface.

OSTEOCLASTS are multinucleated cells responsible for bone resorption. When actively resorbing they are polarized and have three specialized cell membrane areas. Resorption of bone takes place in the ruffled border area, where protons and proteases are secreted into the resorption lacuna between the ruffled border membrane of the osteoclast and the bone surface (Baron et al., 1988; Blair et al., 1989; Bekker and Gay, 1990; Sundquist et al., 1990; Väänänen et al., 1990). The ruffled border area is surrounded by a sealing zone, which forms a tight attachment of the cell membrane to the underlying bone surface, thereby isolating the resorption lacuna from the extracellular fluid and permitting the maintenance of a cell-generated pH gradient. Previously we have described the formation of a specific microfilament organization around resorption lacunae in resorbing osteoclasts (Lakkakorpi et al., 1989). Recently we have further characterized the kinetics of this microfilament structure and demonstrated its stepwise formation into the sealing zone area (Lakkakorpi and Väänänen, 1991). The third specialized membrane domain of the active osteoclast, the basolateral membrane, faces the bone marrow and is not in contact with the mineralized bone matrix.

The molecular mechanisms by which osteoclasts attach to the bone surface are not well understood. The vitronectin receptor ($\alpha_v\beta_3$), one member of the integrin superfamily (Hynes, 1987), has been shown to be expressed in os-

teoclasts (Davies et al., 1989; Horton, 1990; Helfrich et al., 1992). Bone resorption by human and chick osteoclasts can be inhibited with antibodies against the vitronectin receptor (Chambers et al., 1986; Horton et al., 1991). Recently it has been suggested that the vitronectin receptor mediates the tight attachment of osteoclasts to bone matrix (Reinholt et al., 1990; Sato et al., 1990), and that osteopontin is the ligand of the osteoclast vitronectin receptor (Reinholt et al., 1990).

Ligand binding of β_3 integrins is mediated via an arginine-glycine-aspartic acid (RGD)[1] peptide sequence within their ligands (Ruoslahti and Pierschbacher, 1986; Hynes, 1987; Ruoslahti and Pierschbacher, 1987). Recently Sato et al. (1990) showed that echistatin, a protein purified from the venom of the saw-scaled viper *Echis carinatus* and containing an RGD sequence, inhibits bone resorption. They also tentatively identified the echistatin receptor in osteoclasts as the vitronectin receptor.

In this study we have examined the effects of synthetic peptides with and without an RGD sequence on resorption activ-

ity and morphology of osteoclasts in vitro. We have also used several mono- and polyclonal antibodies, which recognize different domains of vitronectin receptor, to localize this receptor on osteoclasts at both the light and electron microscopic levels.

A preliminary report of these experiments with the synthetic peptides was presented at the Third International Workshop of Cells and Cytokines in Bone and Cartilage, Davos, Switzerland, 8–11 April 1990.

Materials and Methods

Isolation and Culture of Osteoclasts and Determination of the Resorption Activity

Procedures for the culture of osteoclasts on bone slices were slightly modified from the original methods of Boyde et al. (1984) and Chambers et al. (1984) and have been described previously in detail (Lakkakorpi et al., 1989; Lakkakorpi and Väänänen, 1990). Briefly, osteoclasts that had been mechanically harvested from long bones of 3–4-d-old rat pups or 3–6-d-old chicks by curetting the endosteal surface with a scalpel blade were allowed to attach to transverse slices (100–150 μm thick) of bovine cortical bone. After 30 min (rat) or 100 min (chicken) nonattached cells were washed away and the cells on bone slices were cultured in DME buffered with 20 mM HEPES and containing 0.84 g/liter sodium bicarbonate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS, pH 6.9, at 37°C and 5% CO_2/95% air for 1 d. Studied peptides were added to the medium after cells were allowed to attach to bone slices.

To determine the resorption activity, bone slices were fixed with 3% paraformaldehyde and 2% sucrose in PBS, pH 7.1, for 5 min and stained with 1% toluidine blue in 1% sodium borate. Bone slices were screened with a 25× objective to count multinucleated osteoclasts. The cells were then removed by ultrasonication in 0.25 M ammonium hydroxide and the resorption pits were counted with phase contrast optics. The resorption index for each bone slice was assessed as the number of resorption pits per osteoclast number (Sundquist et al., 1990). For comparison of data from different experiments, the resorption index of control was used as 100%.

We have shown earlier that the multinucleated cells in these cultures are osteoclasts since they express tartrate-resistant acid phosphatase, actively resorb bone during the culture period, and respond to calcitonin with rapid cytoplasmic retraction and changes in microfilament organization (Lakkakorpi et al., 1989; Lakkakorpi and Väänänen, 1990).

Synthetic Peptides

Pentapeptide glycine-arginine-glycine-aspartic acid-serine (GRGDS) and tripeptide RGD were purchased from Sigma Chemical Co. (St. Louis, MO). Pentapeptide glycine-arginine-glycine-glutamic acid-serine (GRGES) was synthetized with an automated synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) using t-Boc chemistry and purified preparatively using HPLC, and the composition was verified by amino acid sequencing at the Department of Biochemistry, University of Oulu (Oulu, Finland).

Antibodies

Several mono- and polyclonal antibodies against the vitronectin receptor, $\alpha_v\beta_3$, were used to localize the receptor at both light and electron microscopic levels. The rabbit polyclonal antibody purchased from Telios Pharmaceuticals, Inc. (San Diego, CA) recognizes both the α_v and β_3 (and possibly β_5) subunits of the entire vitronectin receptor complex and was used at 1:50 dilution (anti-VNR). Polyclonal anti-β_3 and anti-α_v were a kind gift of Dr. E. Ruoslahti (Cancer Research Center, La Jolla, CA). The anti-β_3 antiserum was raised against a synthetic peptide encompassing the entire cytoplasmic domain of the β_3 chain and the anti-α_v antiserum against an intracellular COOH-terminal fragment of α_v chain (Freed et al., 1989). They were both used at 1:50 and 1:100 dilutions. Polyclonal RB1 antibody (Nesbitt et al., 1989) recognizes the intracellular COOH-terminal nonapeptide of the α_v chain and was used at 1:20 dilution. Monoclonal antibodies F4 and F11 recognize the extracellular domain of the β_3 subunit (Helfrich

et al., 1992) and were used as tissue culture supernatants. Monoclonal antibody 23C6 (Horton et al., 1985) recognizes the intact $\alpha_v\beta_3$ complex of the vitronectin receptor and was also used as neat tissue culture supernatant. Rhodamine-conjugated rabbit anti-mouse, rhodamine-conjugated swine anti-rabbit, swine anti-rabbit, and rabbit anti-mouse Ig were purchased from DAKOPATTS A/S (Glostrup, Denmark) and used at 1:50, 1:20, 1:20, and 1:20 (for peroxidase stainings) or 1:100/1:150 (for immunoelectron microscopic [IEM]) dilutions, respectively. Rabbit and mouse peroxidase-antiperoxidase complex were also purchased from DAKOPATTS A/S. 10-nm protein A-gold complexes were a gift from Dr. Raija Sormunen (Department of Pathology, University of Oulu, Oulu, Finland), and were prepared according to Slot and Geuze (1985).

Immunoperoxidase and Immunofluorescence Stainings

Paraffin sections from Carnoy fixed 17-d-old rat bone were stained by the peroxidase-antiperoxidase technique (Sternberger, 1979). Sections were treated first with 1% BSA in PBS for 5 min and then with 10% swine serum (for polyclonal primary antibodies) or 10% rabbit serum (for monoclonal primary antibodies) in PBS for 60 min. Sections were then incubated with the primary antibody overnight at 4°C, washed for 30 min in PBS, and incubated with swine anti-rabbit Ig diluted 1:20 in 10% swine serum in PBS (for polyclonal primary antibodies) or with rabbit anti-mouse Ig diluted 1:20 in 10% rabbit serum in PBS (for monoclonal primary antibodies) for 30 min. After a 30-min wash with constant stirring in PBS, sections were incubated with rabbit or mouse peroxidase-antiperoxidase complex diluted 1:100 in PBS for 30 min. After a further 30-min wash in PBS the peroxidase reaction was performed with H_2O_2 and diaminobenzidine for 5 min. Finally, sections were washed with deionized water and mounted in glycerol.

For immunofluorescense microscopy, osteoclasts cultured on bone slices were rinsed with PBS, fixed with 3% paraformaldehyde and 2% sucrose in PBS for 5 min at room temperature, rinsed in PBS, permeabilized in Hepes-Triton X-100 buffer (20 mM Hepes, pH 7.0, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X-100) for 5 min at 0°C, and finally rinsed in PBS. Bone slices were incubated with primary antibodies for 1 h at 37°C, thoroughly rinsed in PBS, and then incubated in a rhodamine-conjugated secondary antibody for 30 min at 37°C. Before addition of polyclonal primary antibodies, bone slices were incubated with 10% swine serum for 30 min at 37°C; 10% swine serum was also added to the secondary antibody solution. F-actin was stained with 5 U/ml rhodamine-labeled phalloidin (Molecular Probes, Inc., Eugene, OR).

Immunoelectron Microscopy

5-d-old rat pups were killed by decapitation, the distal femurs were quickly dissected out, and slices were cut from the primary spongiosa area under the growth plate at 0°C. Slices were immersed in 2% fresh paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 3 h. Ultracryotomy was performed according to Tokuyasu (1973) with minor modifications. Thin sections of ∼100 nm were prepared on a Nova ultracryotome equipped with a diamond knife (LKB Instruments, Inc., Gaithersburg, MD). The protein A-gold technique (Geuze et al., 1991) was used to localize the vitronectin receptor. After incubation in fluid gelatin at 37°C, grids were washed in PBS containing 0.02 M glycine, incubated with 1% NaBH$_4$ for 10 min, washed in PBS, and further incubated with
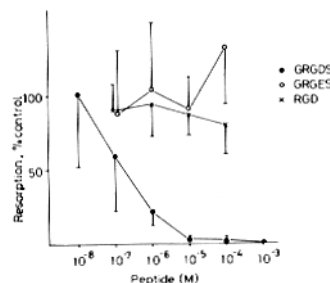
Figure 1. Effects of synthetic peptides on bone resorption by rat osteoclasts. Each peptide was tested in two to four different experiments with three to four different bone slices in each concentration. In control slices 102 ± 33 osteoclasts per bone slice resorbed 0.87 ± 0.43 lacunae/osteoclast in 24 h. GRGDS caused dose-dependent inhibition of resorption with an IC$_{50}$ of 0.1 μM. The values for 10^{-7}–10^{-3} M GRGDS differ significantly from the control ($P < 0.001$). The number of osteoclasts was significantly smaller in the presence of 10^{-3} and 10^{-4} M GRGDS ($P < 0.05$) and 10^{-4} M GRGES ($P < 0.01$) when compared with control.
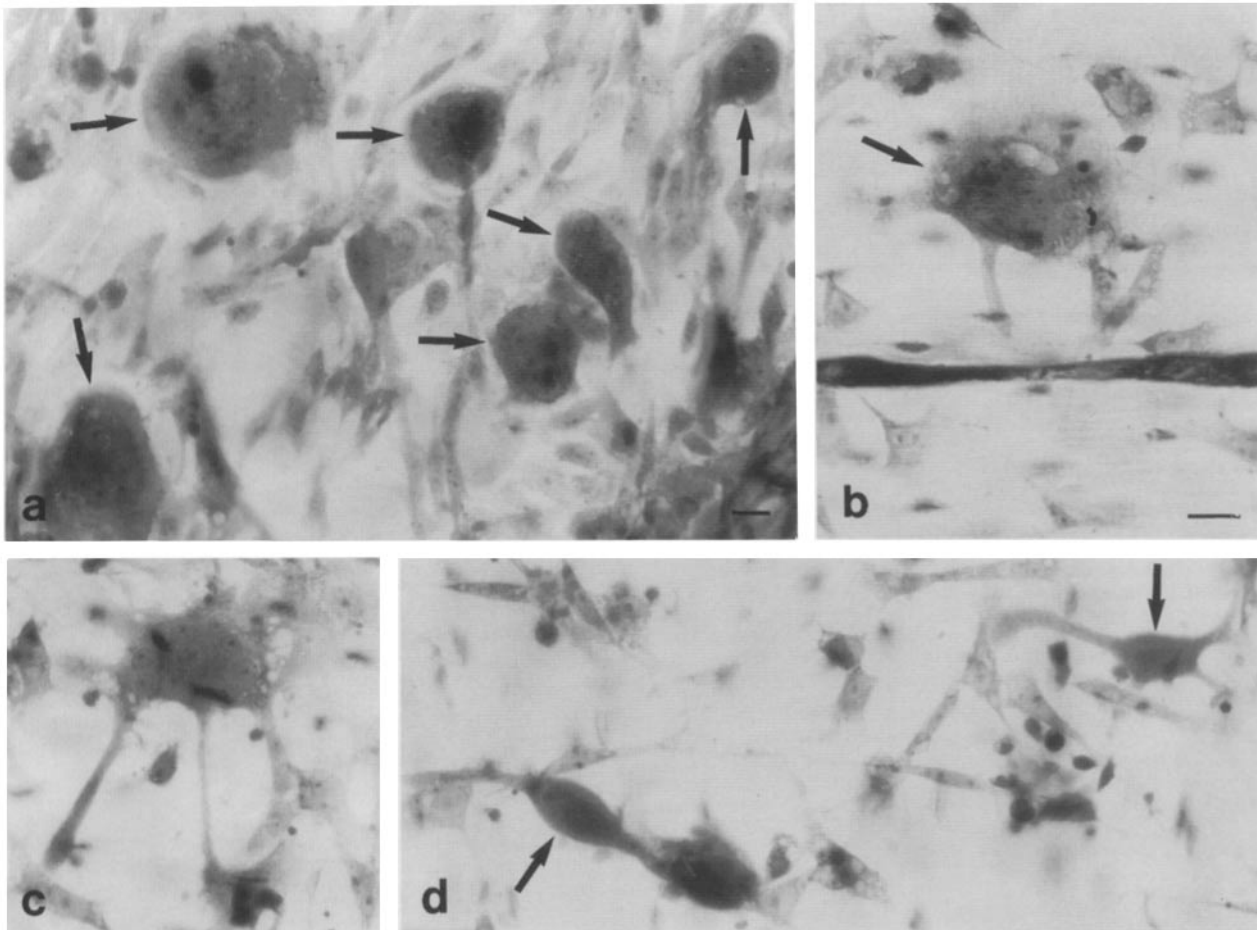
Figure 2. Toluidine blue stainings showing several resorbing (*a, arrows*) and migrating (*b, arrow*) rat osteoclasts in control cultures. Retracted rat osteoclasts with long extensions in the presence of 0.1 mM (*c*) and 1 mM (*d, arrows*) GRGDS peptide. *Bar,* 20 μm.
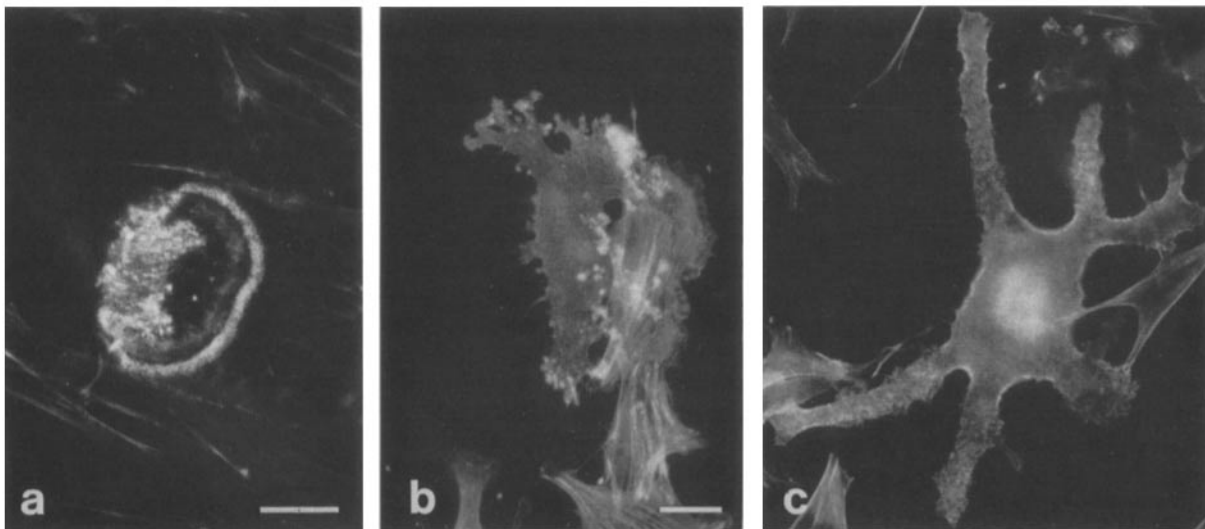
Figure 3. Rhodamine-labeled phalloidin staining of F-actin in rat osteoclasts in control medium (*a* and *b*) and in medium containing 0.1 mM GRGDS peptide (*c*). The figure shows specific ring structure of F-actin at the sealing zone area in resorbing osteoclast in *a* and a typical nonresorbing osteoclast in *b*. Note the different cell shape and diffuse staining of F-actin in the presence of GRGDS peptide (*c*). *Bar,* 20 μm.

normal sheep serum for 30 min. The grids were then incubated with the primary antibody diluted in PBS containing 1% sheep serum for 1 h. When monoclonal antibody was used, grids were incubated with rabbit anti-mouse Ig as a secondary antibody. After thorough washes with PBS, grids were incubated with protein A-gold complexes for 1 h. Finally the grids were washed, stained with uranyl acetate-oxalate, and embedded in 2% methyl cellulose containing 0.3% uranyl acetate. Controls were stained without primary antibody. More than 25 osteoclasts with each antibody were studied under a transmission electron microscope (model 410 LS; Philips Electronic Instruments Co., Mahwah, NJ).

Semiquantitation was performed afterward from pictures. The length of unfractured cell membrane (sealing zone, ruffled border, and basolateral membrane) was measured with an ImageMeasure program (Microscience Inc., Federal Way, WA) using PCVision imaging board (Imaging Technology Inc., Woburn, MA) and the gold particles at the measured line were counted.

Results

GRGDS pentapeptide inhibited bone resorption by isolated rat osteoclasts in a dose-dependent manner over the range 10^{-3}–10^{-8} M when measured by counting the number of resorption pits per osteoclast (Fig. 1). At a concentration of 1 mM, total inhibition of bone resorption was achieved with an IC_{50} of ∼0.1 μM. The number of osteoclasts was significantly reduced in the presence of 0.1 and 1 mM GRGDS peptide. The pentapeptide with aspartic acid replaced by glutamic acid (GRGES) and the tripeptide, RGD, had no significant effect on osteoclast resorption activity (Fig. 1). However, at a high concentration (0.1 mM) of GRGES, the number of osteoclasts adhering to the bone slices was significantly reduced.

GRGDS pentapeptide concentrations of 0.01 mM or more caused clearly visible changes in the shape of rat osteoclasts (Figs. 2 and 3). Osteoclasts were retracted from the bone surface and had long rigid extensions. A concomitant disappearance of the specific microfilament organization at the attachment areas was revealed by actin staining (Fig. 3).

Since the vitronectin receptor is expressed in osteoclasts, it is possible that the interaction of GRGDS peptide with the vitronectin receptor caused the observed changes in osteoclast behavior. To localize vitronectin receptor in osteoclasts we used several antibodies that recognize the α_v or β_3 chain or the whole vitronectin receptor complex. With peroxidase staining of rat bone sections we observed an intense reaction at the cell membrane of osteoclasts with occasional small unstained areas probably corresponding to sealing zones (Fig. 4). This staining of osteoclast cell membranes in paraffin sections was observed with anti-VNR, polyclonal anti-β_3, and RB1 (anti-α_v) antibodies.

Immunofluorescence staining of cultured rat and chicken osteoclasts was performed with all the antibodies listed with similar results. We observed specific staining all over the osteoclast plasma membrane; in resorbing osteoclasts the ruffled border areas over resorption lacunae were most intensively stained. The weakest staining was found around the resorption pits (Fig. 5). In no case was there evidence of increased accumulation of vitronectin receptor around the edges of the resorption pits.

The results obtained at the light microscopic level suggested that the vitronectin receptor in osteoclasts is not polarized to the sealing zone areas, and this finding was confirmed by IEM of rat bone sections. The IEM was carried out with anti-VNR and monoclonal F11 antibodies with similar results. Most of the immunoreaction was at the baso-
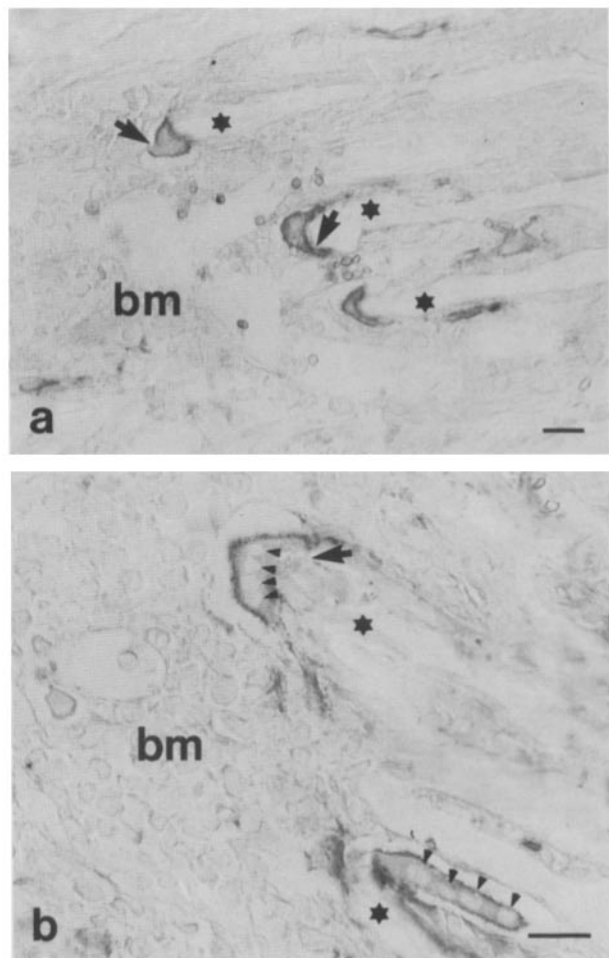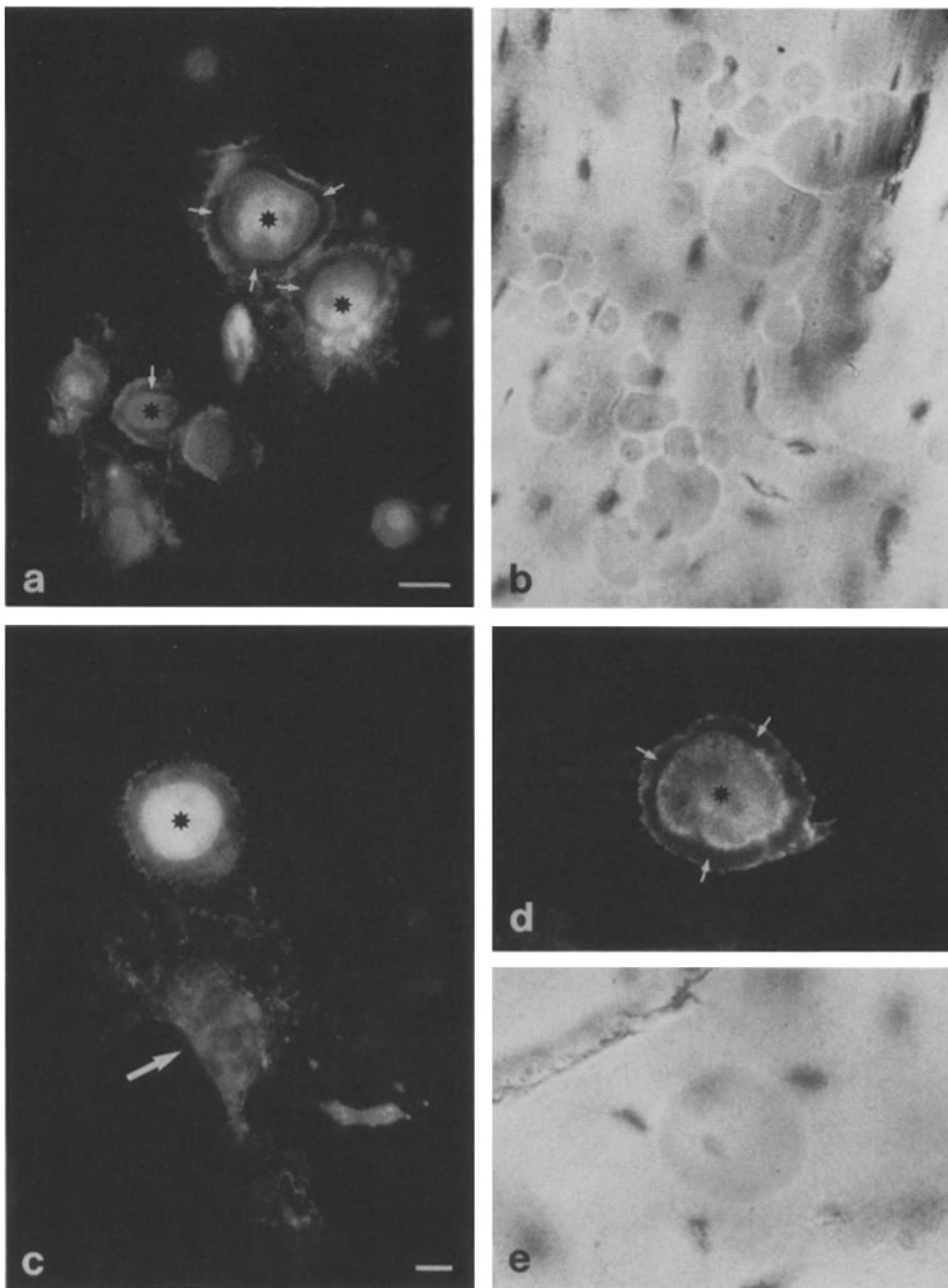

Figure 4. Peroxidase staining of the paraffin section from 17-d-old rat bone with polyclonal anti-β_3 antibody, which recognizes the intracellular domain of the β_3 chain (a), and with polyclonal RB1 antibody, which recognizes the intracellular COOH-terminal peptide of the α_v chain (b). The figure shows intense staining at the cell membrane of osteoclasts with occasional small unstained areas (arrows). Osteoclasts are attached to the bone trabeculae (asterisks) and surrounded by bone marrow (bm). Several nuclei can be seen in osteoclasts in b (arrowheads). Bars, 40 μm (a) and 20 μm (b).

lateral membrane (Fig. 6, a and d); the ruffled border was also clearly stained (Fig. 6 b). At the sealing zone we could not find any indication of specific staining (Fig. 6 c). In bone matrix there was some nonspecific staining, but otherwise the controls showed only minimal staining (Fig. 6 e). The immunoreactivity was semiquantified with the anti-VNR antibody (Table I). The highest density of gold particles (2.43 gold particles/μm of membrane) was found in the basolateral membrane, and the ruffled border membrane and sealing zone membrane showed only 34 and 2% of that density, respectively. The frequency of gold particles at the sealing zone was at the same level in anti-VNR antibody-stained and control osteoclasts.

Discussion

In this study we describe the effects of different synthetic peptides on osteoclast behavior in vitro. The pentapeptide, GRGDS, inhibited bone resorption in a dose-dependent

Figure 5. Immunofluorescence staining of cultured rat osteoclasts with monoclonal F4 antibody, which recognizes the extracellular domain of the β_3 chain (*a*), and with polyclonal anti-β_3 antibody, which recognizes the intracellular domain of the β_3 chain (*c*), and of chicken osteoclast with monoclonal antibody 23C6, which recognizes the $\alpha_v\beta_3$ vitronectin receptor complex (*d*). The figure shows the normal light microscopic picture of corresponding resorption lacunae under the osteoclasts (*b* and *e*). There is intense staining at the ruffled border areas over resorption pits (*a*, *c*, and *d*, *asterisks*) and there are dark areas with very faint staining at the sealing zone areas (*a* and *d*, *arrows*) around the resorption pits (*b* and *e*). In the nonresorbing, probably migrating, osteoclast (*c*, *large arrow*) there is a faint staining all over the cell membrane. *Bars*, 20 μm (*a* and *b*) and 10 μm (*c–e*).

manner with an IC_{50} of 0.1 μM. RGD tripeptide had no statistically significant effect on resorption activity at the concentration tested. It has been previously reported that the RGD tripeptide is inactive in inhibition of cell adhesion at the doses tested; COOH-terminal amidation of the RGD tripeptide or addition of flanking amino acid sequences was required for activity against integrin receptors (Pierschbacher and Ruoslahti, 1987). Sato et al. (1990), using a similar resorption assay with rat osteoclasts, have recently shown that the IC_{50} of the tetrapeptide arginine-glycine-aspartic acid-serine (RGDS), is 100 μM and that of echistatin, 0.1 nM. These results, when taken with our data, clearly show that in addition to the presence of the RGD sequence, the correct conformation at the RGD peptide region is also important for maximal inhibitory activity. Moreover, substitu-

tion of one amino acid in the RGD sequence causes the loss of the inhibitory effect, since pentapeptide GRGES does not inhibit bone resorption. A similar result was obtained with echistatin by changing the arginine to alanine in its RGD sequence (Sato et al., 1990).

GRGDS concentrations of 0.1 mM or more caused clear changes in osteoclast morphology. Our data agree with the earlier findings that higher peptide concentrations are needed for visible morphological changes than for the inhibition of resorption (Sato et al., 1990). Retraction of osteoclasts probably leads in some cases to cellular detachment from bone surface, thus explaining the reduction of the number of osteoclasts with high concentrations of GRGDS peptide. The number of osteoclasts was also reduced in the presence of 0.1 mM GRGES peptide without any specific
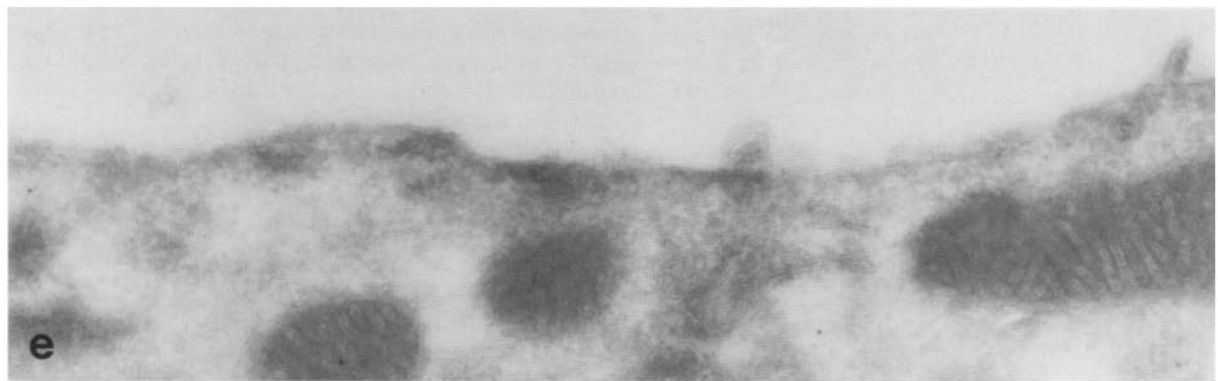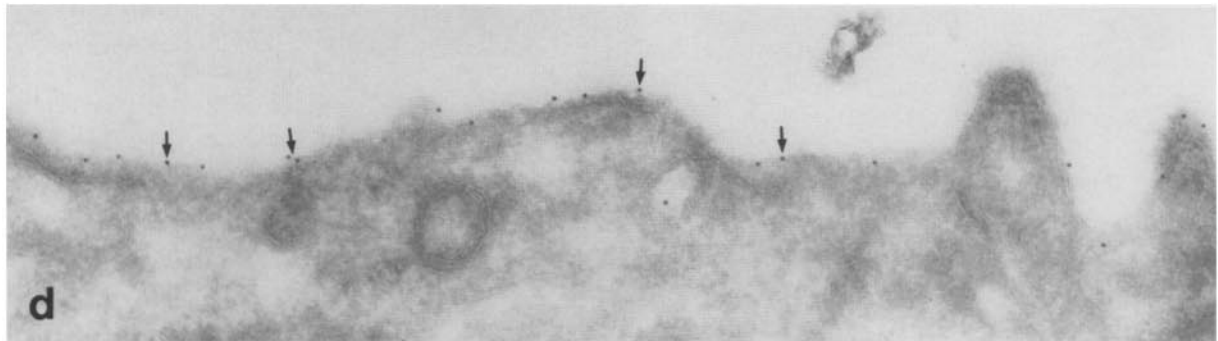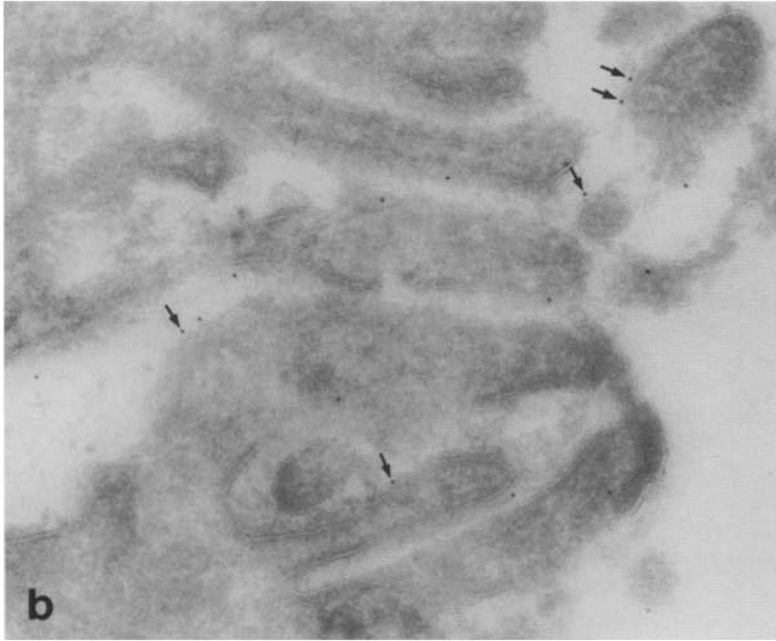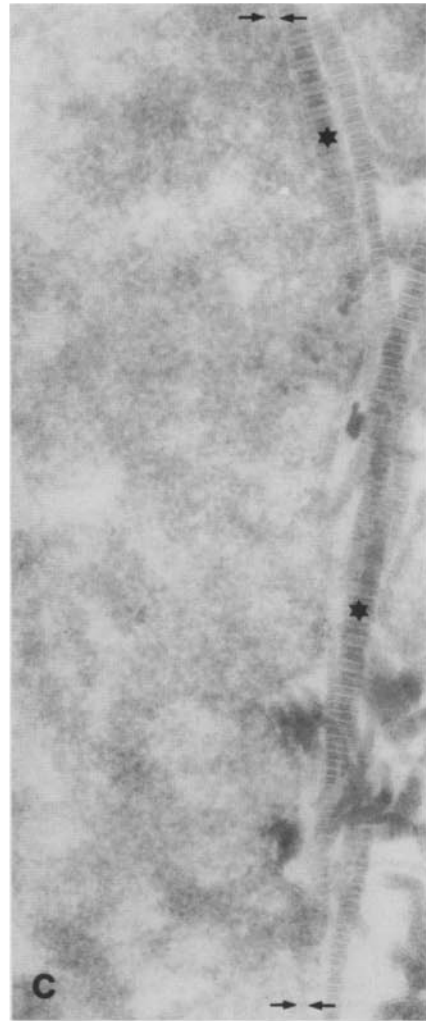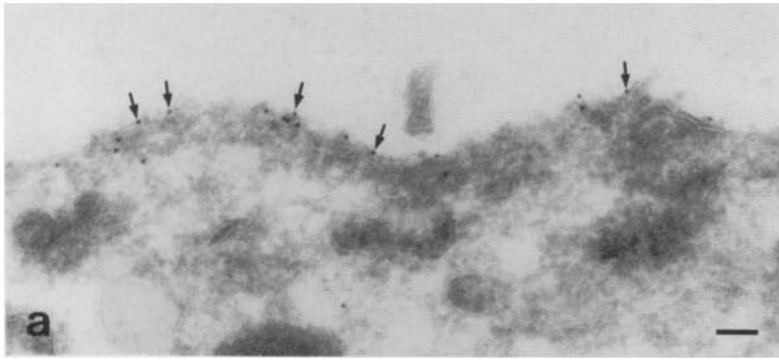
	Number of gold particles per micrometer of membrane		Total length of membrane measured	
	Anti-VNR	Control	Anti-VNR	Control
			μm	
Sealing zone	0.06	0.05	18.0	21.1
Ruffled border	0.82	0.10	261.4	209.1
Basolateral membrane	2.43	0.16	141.4	43.4

Nine and five osteoclasts were measured in anti-VNR and control stainings, respectively.

effect on resorption activity. The explanation for this phenomenon is unclear, though in a separate study (Horton et al., 1991) high doses (400 μM) of a slightly different peptide, glycine-arginine-glycine-glutamic acid-serine-proline (GRGESP), reduced osteoclastic resorption. Similar findings have been reported in other cell systems involving different integrin receptors. Arginine-glycine-glutamic acid (RGE) peptides are, in fact, weak antagonists (1,000-fold less active) when assayed in solid phase receptor assays for fibronectin receptor (Hautanen et al., 1989). They are generally ineffective in cell binding assays (Pierschbacher and Ruoslahti, 1987), although inhibitory activity has been reported in some cell systems involving collagen and other integrin receptors (Grinell et al., 1989; Lawrence et al., 1990).

Because of the restricted expression of β_1 integrins in osteoclasts, by immunocytochemical techniques (Horton and Davies, 1989) only evidence for expression of the RGD-independent collagen/laminin receptor $\alpha_2\beta_1$ (Wayner et al., 1988; Elices and Hemler, 1989) was found. The receptor for the RGD-containing peptides on osteoclasts is probably the vitronectin receptor, which does require an RGD sequence to bind its ligand (Pytela et al., 1985). When we localized the vitronectin receptor in osteoclasts with several antibodies at both light and electron microscopic levels intense cell membrane staining was observed with all the techniques used. Semiquantitation of the immunoreactivity in IEM showed that all specific staining was either at the basolateral membrane or at the ruffled border (2.43 and 0.82 gold particles/μm of membrane, respectively). The level of the staining at the sealing zone was similar in antibody-stained and control sections (0.05 and 0.06 gold particles/μm of membrane, respectively). This, together with our immunofluorescence data, strongly argues against a proposed hypothesis that the tight attachment of osteoclast sealing zone membrane to bone matrix is mediated by the vitronectin receptor (Reinholt et al., 1990). Staining with antibodies against α_v and β_3 chains gave similar results, suggesting that both vitronectin receptor subunits are coexpressed, and colocalized, in osteoclasts. The extensive convolutions of the cell membrane in the ruffled border intensified the staining at the

light microscopic level and thus the brighter staining was seen over resorption lacunae when the immunofluorescence staining method was used. It is possible that ligand occupancy of the vitronectin receptor might prevent the binding of antibodies to the extracellular domain of the receptor; because of this possibility we also used antibodies that recognize the intracellular domain of the α_v and β_3 subunits that would be unaffected by ligand binding. These antibodies gave results similar to the others, strengthening the suggestion that the vitronectin receptor is lacking at the sealing zone membrane. Antibody 23C6, which recognizes the $\alpha_v\beta_3$ vitronectin receptor complex in chicken osteoclasts, also gave similar results, indicating that the observed distribution of vitronectin receptor is not restricted to rat osteoclasts.

These data, as well as the results of others (Chambers et al., 1986; Horton et al., 1991), indicate that vitronectin receptor has an important functional role in bone resorption. However, in contrast to some earlier studies (Reinholt et al., 1990), we could not demonstrate vitronectin receptor at the sealing zone membrane, suggesting that tight attachment of osteoclasts to bone surface is not mediated by this integrin. The colocalization of vitronectin receptor and vinculin in podosome-like structures described by Zambonin-Zallone et al. (1989) may indicate its role in the attachment of resting and migrating osteoclasts to bone surface. The fact that we could not detect the vitronectin receptor at the sealing zone membrane is in agreement with our earlier findings that the association of actin and vinculin seen in podosomes is clearly different in the sealing zone area (Lakkakorpi et al., 1989; Lakkakorpi and Väänänen, 1991). Vinculin is also lacking from the most central area of the sealing zone, indicating that some organization other than a high density of podosomes mediates the attachment of resorbing osteoclasts to bone surface. Thus the exact nature of the molecular mechanism responsible for the formation of tight attachment between the bone surface and the sealing zone membrane of the resorbing osteoclasts remains unclear. Whether this relates to the exclusion of the vitronectin receptor from the membrane to allow close apposition of membrane to bone, forming an ion-impermeable seal, or to the involvement of a further class of receptors remains to be established. By analogy with other cell types (Springer, 1990), adhesion of osteoclasts, their migration over the bone surface, and their formation of a tight sealing zone may be processes that are controlled by different classes of receptors interacting with their ligands over different distances between cell and matrix.

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Figure 6. Immunoelectron microscopic localization of vitronectin receptor in rat osteoclasts with a monoclonal Fll (*a*) and anti-VNR (*b–d*) antibody. The basolateral membrane shows strong staining with both antibodies (*a* and *d*, *arrows*). Clear staining was also revealed at the ruffled border (*b*, *arrows*), but there were no gold particles at the sealing zone membrane (*c*, *arrows*). Collagen fibers can be seen in the bone matrix (*c*, *asterisks*). Only minimal staining was seen inside the osteoclasts or at any membrane areas in control sections (*e*, basolateral membrane). Bar, 0.1 μm.

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