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Spatial organization of microfilaments and vitronectin receptor, $\alpha_v\beta_3$, in osteoclasts

A study using confocal laser scanning microscopy

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SUMMARY

The primary function of the osteoclast is that of the major cell mediating bone resorption. They are actively migrating cells but during resorption they polarize to form a specialized tight attachment structure, the sealing zone, adjacent to the mineralized bone matrix. The processes of adhesion to, and migration on, bone involves cell adhesion molecules, integrins, interacting with their ligands in bone. We have used confocal microscopy to analyse, in rat osteoclasts cultured on bone and glass substrata, the distribution of vitronectin receptor, the major integrin of osteoclasts, and cytoskeletal proteins that it may be linked to. Double staining for F-actin and vinculin, and for vinculin with talin, revealed that cytoskeletal organization differs at various activation states of osteoclasts. Microfilament structures were flat, of 1-5 μm size, and concentrated near the bone surface. The vitronectin receptor was localized both in the basolateral membrane (away from the bone surface) and in the ruffled border (adjacent to bone) in osteoclasts cultured on bone, but was detected mainly in the basolateral membrane when cultured on glass. The vitronectin receptor appeared to be condensed on small microvilli-like projections on the basolateral membrane of osteoclasts on either bone or glass and may provide a route for alternative signalling pathways to modify osteoclast behaviour, other than by influencing cell adhesion directly. The leading edges of migrating osteoclasts, and the attachment structure, a broad vinculin band, which forms before bone resorption, also expressed vitronectin receptor, particularly when the antibody against the α_v subunit was used. F-actin-stained central part of the sealing zone, which is between a vinculin "double circle", failed to be stained with any of the vitronectin receptor antibodies used. These results suggest that the vitronectin receptor is involved in migration and the initial attachment of osteoclasts to the bone surface, but that at the sealing zone there is some other, thus far unknown, mechanism which mediates the tight attachment of the osteoclast cell membrane to bone. Differences in staining for α_v and β_3 were observed, expression of the two chains of vitronectin receptor not being totally coincident. Whilst this could be caused by immunochemical differences between the antibodies used, the finding also implies that novel integrin dimer combinations may exist in osteoclasts.

Key words: confocal microscopy, osteoclast, microfilaments, vitronectin receptor, bone resorption

INTRODUCTION

Osteoclasts are multinucleate, highly differentiated cells, whose primary function is bone resorption. When active, they are polarized and form specialized cell membrane areas, a ruffled border and a sealing zone, facing the mineralized bone matrix. Resorption of bone takes place under the ruffled border, a highly convoluted membrane area. Acid 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 is surrounded by the sealing zone, the organelle-free area rich in actin filaments, which forms a tight attachment of the cell membrane to the bone surface, thereby isolating the specific microenvironment in the resorption lacuna. The third specialized membrane domain of the active osteoclast is the basolateral membrane, which faces away from the bone surface towards the marrow space and is not in contact with the mineralized bone matrix.

Osteoclasts are actively migrating cells, the resorption and migration phases alternating in their life cycle (Kanehisa and Heersche, 1988). The specialized ruffled border

and sealing zone areas appear in osteoclasts only during the resorption phases and disappear in the course of migration. Osteoclasts cultured on glass or plastic form attachment structures, called podosomes, in which an F-actin core is surrounded by a small circle or rosette containing vinculin and talin (Marchisio et al., 1984). Podosomes have also been found in osteoclasts cultured on bone (Zambonin-Zallone et al., 1988; Turksen et al., 1988), and according to our earlier results they represent the primary attachment of osteoclasts to bone surface. Accumulation of podosomes precedes bone resorption (Lakkakorpi and Väänänen, 1991). However, in actively resorbing osteoclasts the microfilaments are reorganized in a specific ring structure, where vinculin and talin form a "double circle" with F-actin sandwiched between (Lakkakorpi et al., 1989). Thus, changes in the migration and resorption phases of osteoclasts are reflected in differences in microfilament structure (Lakkakorpi and Väänänen, 1991). Similar F-actin bands as seen in vitro have also been observed in vivo in bone-resorbing osteoclasts on the endocranial surface of growing calvaria (Kanehisa et al., 1990). Recent electron microscopic studies have also revealed, in addition to the proper sealing zone, so-called irregular sealing zones and sealing zone-like structures, which may be involved in the migration or the initial formation of resorption lacuna (Domon and Wakita, 1991). Taylor and coworkers (1989), in a confocal microscopic study of osteoclasts cultured on dentine, described four different patterns of vinculin staining in chicken osteoclasts and showed that the area of adherence in the resorbing osteoclast was complex and extended into the resorption lacuna.

The molecular mechanisms by which osteoclasts attach to the bone surface are not well understood. Osteoclasts express the vitronectin receptor ($\alpha_v\beta_3$) (Davies et al., 1989; Horton, 1990; Lakkakorpi et al., 1991; Helfrich et al., 1992a) and the collagen receptor $\alpha_2\beta_1$ (Horton and Davies, 1989), both of which are members of the integrin superfamily. It has also been suggested from immunocytochemical analysis that osteoclasts may express $\alpha_4\beta_1$ (Quinn et al., 1991), though we have failed to confirm this finding biochemically (Nesbitt S. A. and Horton M. A., unpublished observations). Integrins are $\alpha\beta$ heterodimeric membrane receptors involved in cell-cell and cell-matrix adhesions (Hynes, 1987). Ligand binding of β_3 integrins is mediated via an arginine-glycine-aspartic acid (RGD) peptide sequence within their ligands (Ruoslahti and Pierschbacher, 1986, 1987; Hynes, 1987). Rat osteoclasts have been shown to utilize vitronectin receptor to bind to a wide range of extracellular matrix proteins, including bone sialoproteins, in an RGD-dependent manner (Helfrich et al., 1992b). Recently it has been shown that peptides containing the RGD sequence inhibit bone resorption and change the morphology of osteoclasts (Sato et al., 1990; Horton et al, 1991; Lakkakorpi et al., 1991). β_3 Integrin has been found to colocalize with vinculin and talin in the podosomes of osteoclastoma giant cells cultured on glass (Zambonin-Zallone et al., 1989). It has been suggested that the vitronectin receptor may mediate the tight attachment of osteoclasts to bone matrix and that osteopontin is the ligand of the osteoclast vitronectin receptor (Reinholt et al., 1990). Our own immunoelectron microscopic studies, however, suggest that although the vitronectin receptor is important in the function of osteoclasts, it is located only at the ruffled border and basolateral membranes but is missing from the area of the tight sealing zone (Lakkakorpi et al., 1991).

In the present study we have characterized, by confocal microscopy, the microfilament arrangement and the localization of the vitronectin receptor and their relative spatial distributions in osteoclasts cultured on glass and their natural substratum, mineralized bone.

MATERIALS AND METHODS

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, which had been mechanically harvested from long bones of 2- to 4-day-old rats 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 minutes, non-attached cells were washed away and the cells on bone slices were cultured for one or two days in Dulbecco's Modified Eagle's Medium buffered with 20 mM HEPES and containing 0.84 g/l sodium bicarbonate, 2 mM L-glutamine, 100 i.u./ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum, pH 6.9, at 37°C and 5% CO_2/95% air. In some experiments osteoclasts were cultured on glass coverslips for three days. We have shown 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). 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 with secondary antibodies 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. A monoclonal mouse antibody against chicken gizzard smooth muscle vinculin was purchased from ICN Immuno Biologicals, and used at 1:50 dilution. Rabbit anti-talin serum was a kind gift from Dr. Keith Burridge (University of North Carolina, Chapel Hill, North Carolina, USA) and used at 1:50 dilution. Several antibodies against vitronectin receptor, $\alpha_v\beta_3$, were used. The rabbit polyclonal antibody purchased from Telios Pharmaceuticals Inc. (San Diego, CA) recognizes external and cytoplasmic domains of both the α_v and β_3 (and possible β_5) subunits of the vitronectin receptor complex and was used at 1:50 dilution (anti-VNR). Polyclonal anti-β_3 and anti-α_v were kind gifts from 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 C-terminal fragment of α_v chain (Freed et al., 1989). They were used at 1:50 and 1:100 dilutions. Monoclonal antibody F11 recognizes the extracellular domain of the rat β_3 subunit (Helfrich et al., 1992a) and was used as neat tissue culture supernatant. Rhodamine-conjugated rabbit anti-mouse and swine anti-rabbit immunoglobulins (Dakopatts A/S,

Glostrup, Denmark), and fluorescein-conjugated goat anti-mouse immunoglobulins (Jackson ImmunoResearch Laboratories, Inc., Pennsylvania, PA, USA), were used as secondary antibodies with the dilutions of 1:100, 1:100 and 1:200, respectively. F-actin was stained with fluorescein-labeled phalloidin (Molecular Probes, Inc.), at 5 units/ml. Bone slices were mounted in PBS-glycerol and viewed with a confocal laser scanning microscope, consisting of a Wild Aristoplan fluorescence microscope and Leica Lasertechnik GmbH 1.05 software (Heidelberg, Germany) equipped with a multiline 750 mW air-cooled Omnichrome argon-crypton laser (Chino, California, USA), where 488 and 568 nm lines can be selected. Bone slices were viewed with 50×, 63× and 100× immersion objectives with appropriately chosen pinholes using 256 × 256 image format. The microscope has a computer-controlled stage moveable in the *z*-direction with a 40 nm resolution. Step sizes of 0.21-0.50 μm for *xy*-scans and of 0.49 μm for *xz*-scans were used; other intervals are stated in the figure legends. Resorption lacunae were visualized with nonconfocal transmission illumination. Altogether about 350 osteoclasts were investigated by conventional fluorescence microscopy and 55 of them were scanned through with the confocal microscope. The photographs were taken directly from the monitor after computer processing of the data and generation of pseudocolour images where appropriate.

RESULTS

Fig. 1 shows double staining for F-actin and vinculin and for vinculin with talin in osteoclasts which are at different phases of the resorption cycle. In non-resorbing, presumably migrating, osteoclasts there were either areas of F-actin and vinculin staining with a podosome-like structure (Fig. 1A) or organization into a broad ring of F-actin and vinculin staining, in which podosomes were also detected (Fig. 1B). In resorbing osteoclasts, under which a resorption lacuna could be found, vinculin formed a "double circle" and F-actin a broad zone between the vinculin circles (Fig. 1C). Fig. 1D shows a transmission image of a resorption lacuna underneath the cell illustrated in Fig. 1C which coincides with the centre of the ring microfilament structure.

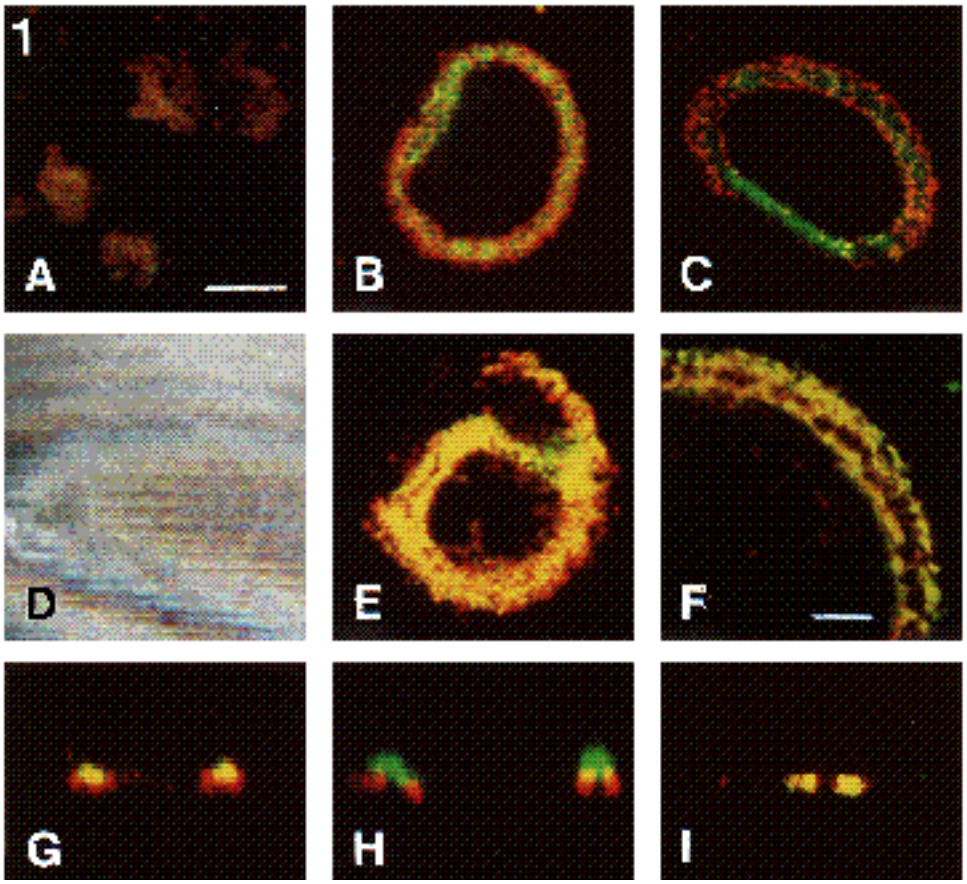

Fig. 1. Pseudocoloured confocal images from double immunofluorescence staining for F-actin (green) and vinculin (red) (A-C, G and H), and for vinculin (green) and talin (red) (E, F and I). Colocalization of the proteins is seen as yellow. (A) An optical section from a non-resorbing osteoclast showing areas of podosomes. (B) An optical section showing a broad ring of podosomes. (C) An optical section from a resorbing osteoclast showing a "double circle" of vinculin with F-actin sandwiched between. (D) Non-confocal transmission image showing the resorption pit in the middle of the ring structure shown in (C). (E) An optical section showing the colocalization of vinculin and talin in a broad ring of podosomes (E) and in the "double circle" structure (F). (G) A cross-section of the broad ring of podosomes computed from 25 optical sections taken at 0.29 μm intervals (i.e. the depth of the whole image was 7.25 μm). (H) A cross-section of the ring structure in resorbing osteoclast in (C) calculated from 25 optical sections taken at 0.29 μm intervals (i.e. the total image depth was 7.25 μm). (I) A cross-section of the "double circle" calculated from 16 optical sections taken at 0.21 μm intervals (i.e. the depth of the image was 3.36 μm). Bars: 10 μm (A-E, G and H); 5 μm (F and I).

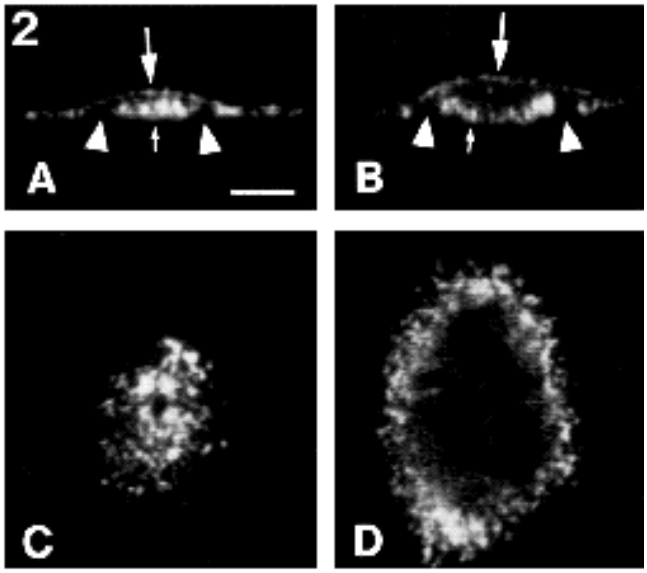

Fig. 2. Confocal images from immunofluorescence staining for vitronectin receptor (anti-VNR). (A and B) Optical sections from *xz*-scanning of resorbing osteoclast at 3.43 μm intervals. Note the staining at the basolateral membrane (large arrows) and ruffled border (small arrows) but absence of staining in the area between (the presumptive sealing zone, arrowheads). (C and D) Optical sections from *xy*-scanning of the top of the cell at 2.5 μm intervals. Note staining is seen on the small surface projections in the basolateral membrane. Bar 10 μm.

Colocalization (shown as a yellow colour formed by fusion of the green and red pseudocolour images) of F-actin with vinculin staining was seen in the areas containing podosomes (Fig. 1A), at the broad vinculin rings (Fig. 1B) and less markedly at the edges of vinculin "double circles" (Fig. 1C). Between the vinculin "double circles" there was a distinct zone containing only F-actin staining (Fig. 1C). Double staining for vinculin and talin were completely colocalized in all forms of microfilament organization (Fig. 1E,F).

xz-Sectioning (Fig. 1G,H,I) in all forms of the microfilament-containing structures examined, showed that vinculin and talin were more closely apposed to the cell membrane than F-actin; all these structures were flat and located near the bone surface. The thickness of the vinculin- and talin-stained structures varied between 1 and 4 μm and of F-actin staining between 1.5 and 5 μm. For F-actin, the zone between the vinculin "double circles" was thickest (3-5 μm), and the thinnest accumulation was seen in the areas of morphologically identifiable podosomes.

All the antibodies against the vitronectin receptor revealed intense staining at the basolateral and ruffled border membranes (as seen in the optical sections in Fig. 2A,B), including the small projections present in the basolateral membrane (Fig. 2C,D). The ruffled border was seen as a zone a few micrometres thick because of extensive convolutions of the ruffled border membrane. By staining for the vitronectin receptor together with vinculin it was possible both to locate the exact position of the sealing zone (the "double circles" of vinculin with the zone of F-actin) and to identify newly formed sealing zones before resorption lacunae could be detected. A central part of the sealing zone (the F-actin-stained area between vinculin "double circles") was unstained with any of the antibodies against the vitronectin receptor (Figs 3, 4). Staining with the anti-α_v antibody partially colocalized at the edges of the vinculin "double circle", but between the vinculin circles we could not find any evidence for α_v expression (Fig. 3). However, anti-α_v antibody staining did colocalize with the broad ring of vinculin expression, which forms preceding bone resorption (Fig. 5). Such colocalization with vinculin was only rarely seen with anti-VNR or anti-β_3 antibodies (Fig. 4). Non-resorbing, presumably migrating, osteoclasts revealed staining for vitronectin receptor at their leading edges, but podosome structures were difficult to discern (Fig. 5B), unlike the findings with microfilament antibodies where podosomes were readily identified.

In osteoclasts cultured on glass, we found that vitronectin receptor staining colocalized with vinculin in podosomes (Fig. 6A,B). As on bone, the podosome rosette structure was not as well defined by vitronectin receptor staining as by vinculin. In addition, all the antibodies against the vitronectin receptor (anti-VNR, anti-α_v, anti-β_3 and the monoclonal F11) stained the cell membrane facing away from the glass surface, particularly on surface villi (Fig. 6C,D).

DISCUSSION

In confocal laser scanning microscopy the pinhole aperture suppresses signals from structures located outside the focal plane. This means that the confocal microscope is an ideal tool to reveal detailed structures in thick samples, such as the resorbing osteoclast whose depth can exceed 25 μm, and that the marked autofluorescence of bone at the emmission wavelengths for fluorescein can be avoided. Therefore, we have been able to carry out double immunofluorescence staining of osteoclasts, whilst they were on their natural substratum, mineralized bone.

Results with double staining for F-actin and vinculin, and for vinculin and talin, support our earlier findings with single immunostaining and conventional fluorescence microscopy (Lakkakorpi et al., 1989; Lakkakorpi and Väänänen, 1991). Cytoskeletal organization varied at the different activation states of osteoclasts, that is during cell migration and bone resorption. We confirmed our earlier demonstration of the existence of a unique microfilament organization in resorbing osteoclasts (Lakkakorpi et al., 1989; Lakkakorpi and Väänänen, 1991). This specific ring structure (a "double circle" of vinculin with F-actin interposed) appears essential for resorption, being found in osteoclasts located over resorption lacunae. The *xz*-series showed that all microfilament structures were flat, ranging from 1 to 5 μm in depth, the F-actin zone in resorbing osteoclasts being the thickest. It also showed that in all the different microfilament arrangements analysed, vinculin and talin were found to be located nearer to the cell membrane than F-actin. Vinculin and talin were always completely colocalized. These results are in agreement with the general assumption that vinculin and talin are located at the ends of the actin filaments. It is presumed that the vinculin

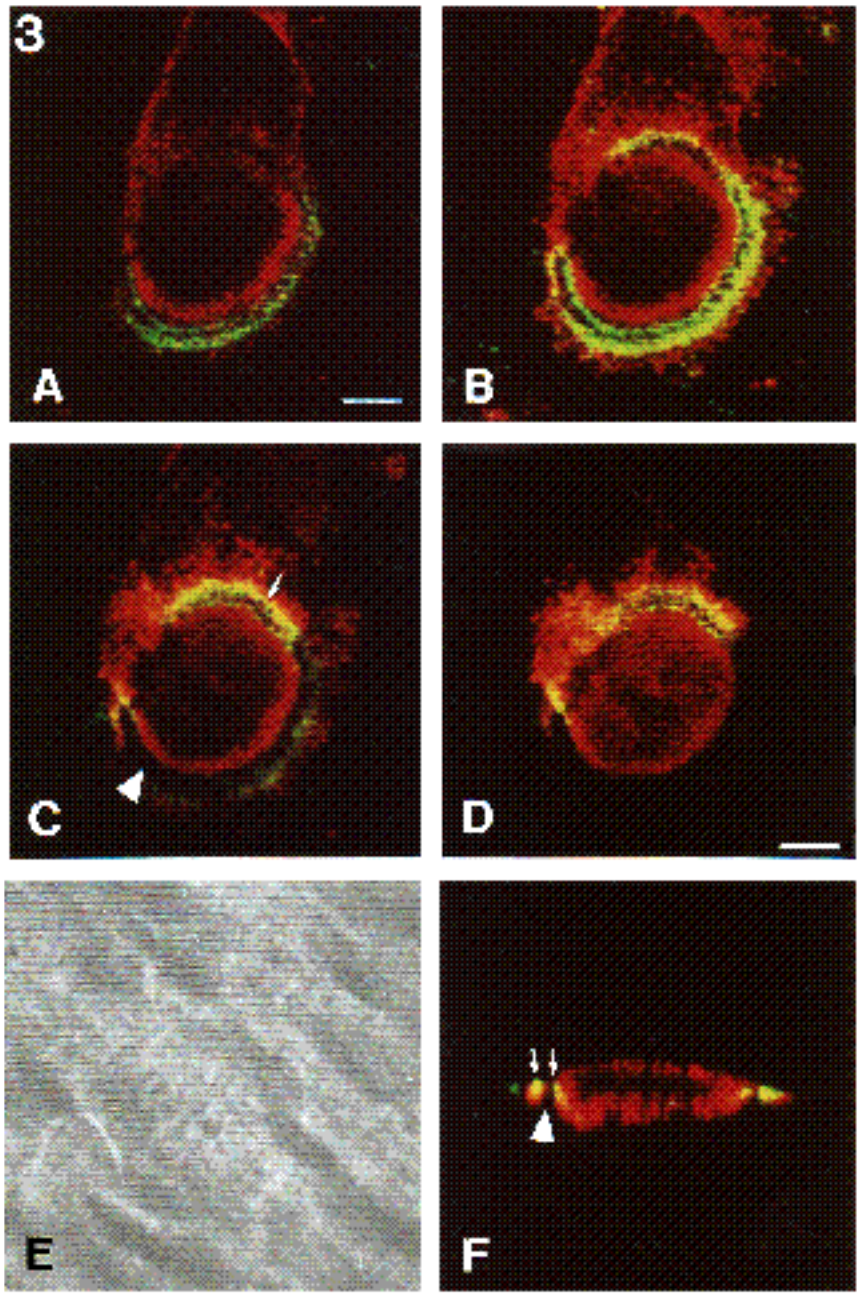

Fig. 3. Pseudocoloured confocal images from double immunofluorescence staining for vinculin (green) and vitronectin receptor (anti-α_v, red). Colocalization of the proteins is seen as yellow. (A-D) Each picture represents one optical section at 1.0 μm intervals towards the bone, the last (D) being below the level of the bone surface. (E) Transmission illumination of the resorption lacuna under the cell shown in A-D. (F) An optical *xz*-section of the same cell. Note that the vitronectin receptor is expressed in the basolateral membrane and the ruffled border deep in the bone but is absent from the sealing zone (C and F, arrowheads). Vinculin is found colocalized with the vitronectin receptor at the edges of the sealing zone that is at the vinculin "double circle" (C and F, arrows). Bar, 10 μm.

"double circle" with F-actin sandwiched between corresponds to the area of tight attachment of the cell to the bone surface, the sealing zone, as observed in transmission electron microscopy.

Vitronectin receptor was found in the leading edges of migrating osteoclasts, in the basolateral membrane, including high levels in surface villi, and in the ruffled border of osteoclasts resorbing bone. Vitronectin receptor distribution in the sealing zone was analysed by double staining for vitronectin receptor and vinculin and *xy*- and *xz*-scanning. In confirmation of our previous immunoelectron microscopy results (Lakkakorpi et al., 1991), vitronectin receptor was undetectable in the F-actin-rich zone between the vinculin "double circle" of resorbing osteoclasts. However, the α_v chain of vitronectin receptor was found at the vinculin "double circle" and colocalized in the broad ring of vinculin which forms before the initiation of bone resorption. These data suggest that the vitronectin receptor, $\alpha_v\beta_3$, is not involved in the formation of the central part of the sealing zone.

The apparent absence of immunostaining for vitronectin receptor in the sealing zone could be due to the binding of ligand to extracellular receptor domains or microfilaments to the cytoplasmic tail of integrins interfering with access of antibodies. In an attempt to avoid such artifacts, we used a range of antibodies to both extra- and intra-cellular parts of α and β chains of vitronectin receptor. Significantly, the condensation of microfilaments into the broad vinculin-rich zone, which precedes bone resorption, or at podosomes, did not prevent staining for vitronectin receptor. In addition, in

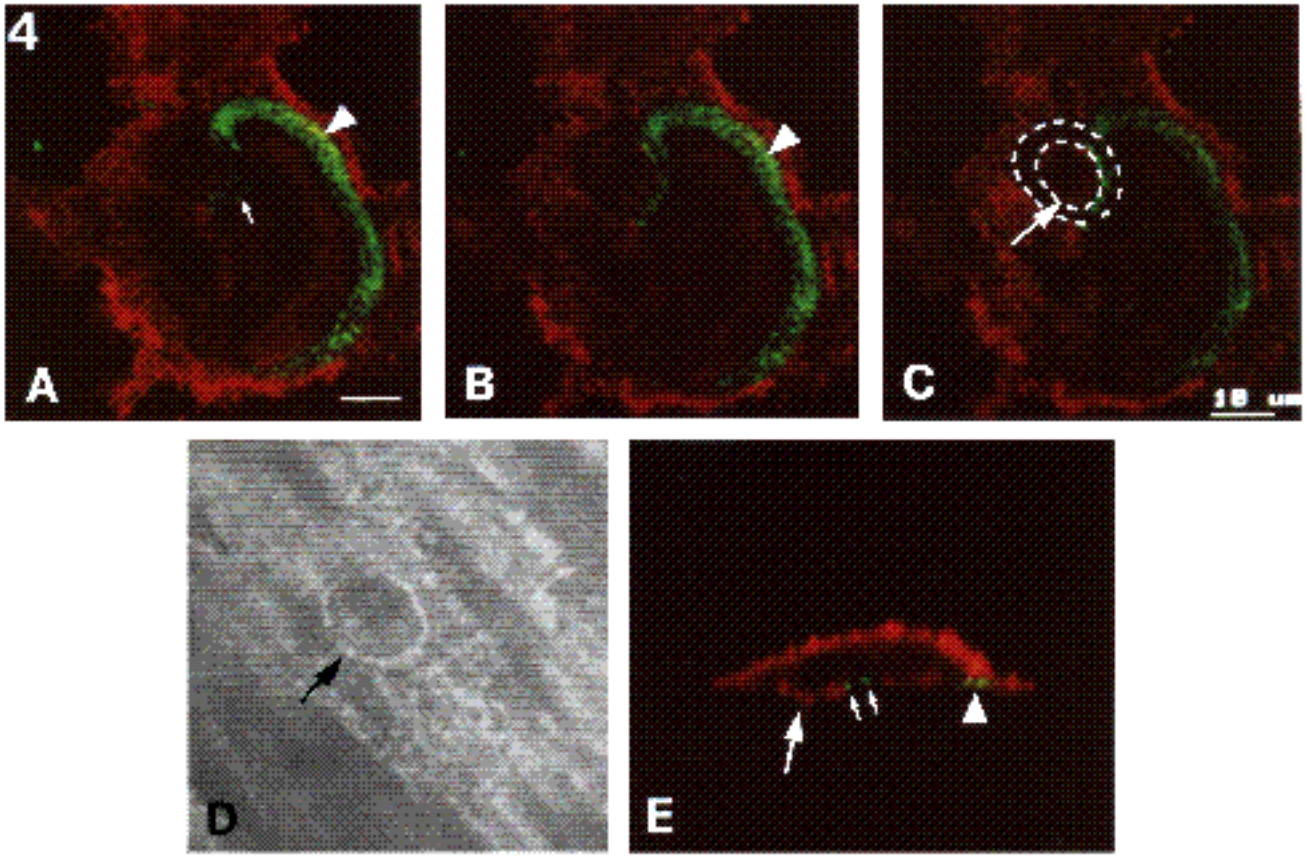

Fig. 4. Pseudocoloured confocal images from double immunofluorescence staining for vinculin (green) and vitronectin receptor (anti-VNR, red). (A-C) Optical sections from *xy*-scanning with 0.5 μm intervals; (D) transmission illumination of the bone; and (E) one optical section from *xz*-scanning of the same cell. Note the broad vinculin area, where the sealing zone is in the process of forming (A, B and E, arrowheads, no resorption pit associated in D) and traces of the "double circle" of vinculin (A and E, small arrows) at the mature sealing zone, which is associated with the resorption pit. Over the resorption pit (D, arrow) there is staining for vitronectin receptor at the ruffled border (C and E, arrows) but not at the sealing zone (C, area between the broken lines); vinculin is detected only at the edge of the sealing zone (A and E, small arrows). Bar, 10 μm.

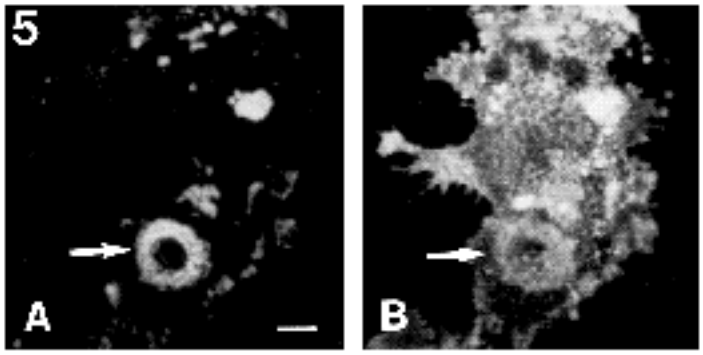

Fig. 5. Confocal images from double immunofluorescence staining for vinculin (A) and vitronectin receptor (anti-α_v; B). Pictures are extended focuses merged from 16 optical sections taken at 0.33 μm intervals. Note a broad ring visualized with both antibodies (arrows) and that podosomes were difficult to detect. Bar, 10 μm.

our earlier study using light microscopy and immunoelectron microscopy of bone sections we were not able to find the vitronectin receptor, $\alpha_v\beta_3$, at the sealing zone (Lakkakorpi et al., 1991). The minor differences observed in the staining pattern for α_v and β_3 could be caused by different affinities of the antibodies or may suggest that α_v and β_3 are, indeed, not totally colocalized in osteoclasts. Taken together, our data imply the existence in osteoclasts of further integrin dimers or other adhesion structures, as yet undefined. Owing to the lack of suitable reagents we have not been able to analyse the distribution of other integrins, such as β_1, in rat osteoclasts.

Peptides containing the RGD sequence inhibit bone resorption, in all probability by interfering with the vitronectin receptor (Sato et al., 1990; Lakkakorpi et al., 1991; Horton et al., 1991). The fact that the vitronectin receptor is involved in the migration of osteoclasts and in the early phases of resorption may explain the inhibition of resorp-

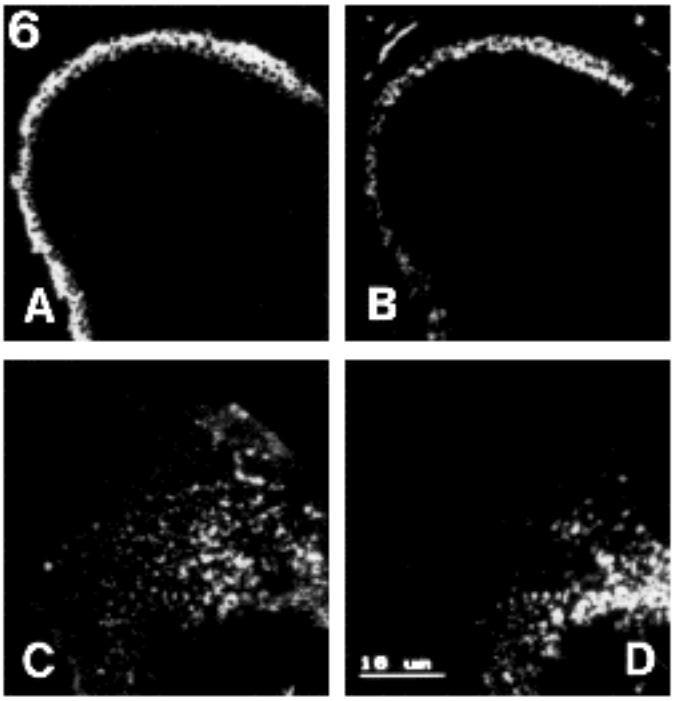

Fig. 6. Confocal images from double immunofluorescence staining for vinculin (B) and vitronectin receptor (anti-VNR, A, C and D) in an osteoclast cultured on glass. (A and B) Extended focuses merged from 6 optical sections taken at 0.21 μm intervals near the glass surface showing distinct podosome structures. (C and D) Optical sections show that the staining was detected in villi over the basolateral membrane, facing away from the glass surface. The distance between (A) and (C) is 1.26 μm, and between (C) and (D) 0.63 μm. Bar, 10 μm.

tion by RGD peptides. When migration and activation of osteoclasts towards its resorptive phase are abolished, the bone resorption process as a whole is inhibited. There is also growing evidence that integrins mediate functions in cells other than cell adhesion and co-operate with other modulators of cell activity (Hynes, 1992). Our finding of high levels of vitronectin receptor in membrane areas not involved in cell-matrix contact, such as the microvilli-like projections of the basolateral membrane, when taken with recent data on signal transduction via vitronectin receptor in osteoclasts (Miyauchi et al., 1991; G. Shankar, I. Davison, M. H. Helfrich, W. T.. Mason and M. A. Horton, unpublished data), also support the concept that the vitronectin receptor may have a more versatile function than merely mediating attachment to the bone matrix.

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