Localization and possible role of two different alpha v beta 3 integrin conformations in resting and resorbing osteoclasts

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Summary

Integrins are membrane receptors that mediate interactions between cells and the extracellular matrix. We recently showed that the osteoclast integrin $\alpha_v\beta_3$ exists in two different conformations, so-called 'basal' and 'activated', with each exhibiting a distinct function. In this study we demonstrate that, in non-resorbing osteoclasts, the 'activated' form of $\alpha_v\beta_3$ accumulates in the motile areas of the plasma membrane. During bone resorption this conformation is prevalent in the ruffled membrane, whereas the 'basal' form of $\alpha_v\beta_3$ is also present in the sealing zone. Moreover, hepatocyte growth factor (HGF) and macrophage colony stimulating factor (M-CSF), two molecules involved in osteoclastogenesis and osteoclast survival, modulate $\alpha_v\beta_3$ conformation in vitro. Pre-incubation with HGF or M-CSF induces a shift of conformation of $\alpha_v\beta_3$ in primary human osteoclasts (OCs) and in the osteoclast-like cell line (GCT 23). Activated integrin promotes osteoclast migration to the $\alpha_v\beta_3$ ligand osteopontin and enhances bone resorption. Thus, HGF and M-CSF modulate the $\alpha_v\beta_3$ conformational states required for osteoclast polarization and resorption. The capacity of growth factors to alter the affinity of $\alpha_v\beta_3$ toward its ligands offers a potential explanation for the diverse responses of osteoclasts to the same ligand.

Key words: Osteoclasts, Alpha v beta 3, Integrin, HGF, M-CSF

Introduction

Integrins are membrane receptors that mediate the interactions between cells and the extracellular matrix. Cellular attachment to immobilized ligands commonly results in cytoskeletal reorganization and clustering of integrins at discrete adhesive sites known as focal contacts or, in osteoclasts (OCs) and cells of monocytic lineage, podosomes (Marchisio et al., 1984). In these specialized structures, an array of submembranous proteins, ranging from structural molecules to regulatory enzymes, form a multimolecular complex linking the actin microfilament network with integrins and, hence, with the extracellular matrix (Miyamoto et al., 1995). The formation of focal contacts or podosomes triggers signaling cascades that, in concert with growth factor-activated transduction pathways, regulate cell proliferation, differentiation, survival and migration (Clark and Brugge, 1995; Schwartz et al., 1995). Integrins adhere to various extracellular matrix proteins, and their affinity for these ligands can be altered by conformational changes in their ligand-binding sites in response to cellular signals (Hughes et al., 1997; LaFlamme et al., 1994). Evidence is accumulating that these 'basal' and 'activated' forms, characterized by different substrate affinities, are functionally distinct. In M21 melanoma cells, the activation of $\alpha_v\beta_3$ integrin results in increased numbers of adherent cells, which bind to the ligand more weakly than the 'basal' integrin, facilitating migration (Pelletier et al., 1996). We recently demonstrated that, similar to the α_3-containing integrins in melanoma cells and platelets (Pelletier et al., 1995), $\alpha_v\beta_3$ also exists in human osteoclast-like cells in both the 'basal' and 'activated' states (Faccio et al., 1998).

The functional cycle of the OC consists of migration towards bone, followed by adherence to the bone surface, where the cell polarizes and initiates the resorptive process. Motile OCs are non-polarized cells, characterized by the presence at their leading edge of membrane protrusions, called lamellipodia, and, in a row behind the leading edge, by podosome complexes, comprising filaments of actin associated with several actin-binding proteins. The passage from a motile to a resorbing cell involves cytoskeletal reorganization and matrix attachment. Following attachment to the bone surface, podosomes assemble in an actin-rich region of the membrane, forming a tight sealing zone, enclosing the resorptive microenvironment. Following insertion of secretory vesicles, a highly convoluted ruffled membrane, the ruffled border, forms facing the bone surface where resorption will take place, while the basosolateral membrane is involved in membrane trafficking (Fig. 4A).

Integrins are believed to play a role in OC activity by mediating matrix adhesion and regulating the cytoskeletal organization required for both cell migration and formation of the sealing zone and ruffled border. OCs express abundant $\alpha_v\beta_3$ integrin (Zambonin-Zallone et al., 1989), which recognizes

bone matrix proteins (Davies et al., 1989; Duong et al., 2000; Lakkakorpi et al., 1991; Nesbitt et al., 1993). Addition of RGD-containing peptides or disintegrins (such as echistatin or kistrin), which bind to $\alpha_v\beta_3$, arrest OC adhesion to bone or cause retraction of attached osteoclasts (King et al., 1994; Nakamura et al., 1996; Nakamura et al., 1999; Sato et al., 1990). Moreover, OCs generated from β_3-deficient mice are dysfunctional in vitro and in vivo (McHugh et al., 2000; Feng et al., 2001).

Recent evidence shows that activation of the $\alpha_v\beta_3$ integrin induces the $[Ca^{2+}]_I$-dependent phosphorylation of Pyk2, a non-receptor tyrosine kinase involved in formation of the sealing zone. Activated Pyk2 forms a complex with c-Src and c-Cbl, and these molecules are all involved in outside-in activation of $\alpha_v\beta_3$ signaling, possibly leading to podosome assembly (Sanjay et al., 2001). Nakamura et al. recently showed that $Src^{-/-}$ prefusion OCs adhered to, but failed to spread on, vitronectin (Vn)-coated surfaces and that $\alpha_v\beta_3$-integrin-mediated signaling was inhibited in these cells. Moreover, the same authors showed that the spreading defect of $Src^{-/-}$ pOCs could be rescued by M-CSF treatment, which resulted in recruitment of the $\alpha_v\beta_3$ integrin to adhesion contacts. However, the authors did not directly assess whether or not M-CSF treatment leads to changes in integrin affinity for ligand.

Cross-talk between integrin-mediated adhesion and growth factors has been described in many recent studies; however, the underlying mechanisms remain incompletely understood. PDGF induces the association of its phosphorylated receptor with integrin $\alpha_v\beta_3$ in fibroblasts plated on vitronectin, which correlates with enhanced PDGF-induced cell proliferation (Schneller et al., 1997). Basic fibroblast growth factor (bFGF) enhances cell migration of vascular endothelial cells, leading to concentration of 'activated' $\alpha_v\beta_3$ integrin to polarized lamellipodia (Kiosses et al., 2001). Moreover, in transformed cells HGF promotes cell migration by increasing $\alpha_v\beta_3$ integrin affinity (Trusolino et al., 2000).

Recently the crystal structure of $\alpha_v\beta_3$ integrin has been solved (Xiong et al., 2001; Beglova et al., 2002), showing that ligand binding itself alters the conformation of this integrin, exposing neo-epitopes, known as ligand-induced binding sites (LIBS). LIBS can also be exposed by intracellular signaling events, a process known as inside-out signaling. Anti-LIBS antibodies can be used as tools to identify integrins in the 'activated' conformation or to convert an integrin from the 'basal' to the 'activated' state. AP5 is an anti-LIBS Ab that recognizes the β_3 N-terminus and is regulated by cation-binding at a site distinct from the LIBS (Honda et al., 1995). At normal extracellular calcium levels, AP5 binds to $\alpha_v\beta_3$ only when the integrin is in the 'activated' conformation. However, in low calcium concentrations AP5 can bind to all forms of the integrin leading to its activation. Once AP5 is bound at low calcium concentrations, $\alpha_v\beta_3$ remains in the 'activated' state even when the calcium levels are increased. It has also been possible to generate monoclonal antibodies that specifically recognize the 'activated' conformation of an integrin at the ligand-binding site. WOW1 is one such antibody, in which the heavy chain hypervariable region 3 of PAC1 Fab was replaced with a single α_v-integrin-binding domain, from the multivalent adenovirus penton base, to engineer an antibody that binds to only activated $\alpha_v\beta_3$ (Pampori et al., 1999). Thus, binding of WOW1 can be used as another marker of integrin activation. In contrast to AP5 and WOW1, AP3 is a monoclonal Ab, which binds to the $\alpha_v\beta_3$ integrin regardless of its conformational state. In this paper we defined the fraction of integrin that is recognized by AP5 and/or WOW-1 as the 'activated' conformation and the fraction of integrin that binds to AP3 and/or 1A2, but not to AP5 or WOW-1, as the 'basal' or 'non-activated' conformation.

This study focuses on the functional implications of $\alpha_v\beta_3$ conformation in human OCs. We utilized different sources of human osteoclasts, giant cell tumors of bone-derived cell line, bone-marrow-derived osteoclasts and osteoclastoma cells, all of which have been shown to express osteoclastogenic markers. We demonstrate distinct localization of the 'basal' and 'activated' integrin in resting and resorbing OCs. We find that during bone resorption 'activated' $\alpha_v\beta_3$ is mainly localized in the ruffled border, whereas the 'basal' conformation is responsible for the adhesive properties of the sealing zone. Moreover we demonstrate that HGF and M-CSF, two growth factors that regulate osteoclastogenesis and osteoclast survival (Fuller et al., 1993; Grano et al., 1996), modulate the activation state of $\alpha_v\beta_3$. Growth factor treatment alters the affinity of $\alpha_v\beta_3$ toward its ligands, promoting OC migration to the $\alpha_v\beta_3$ ligand osteopontin and enhancing bone resorption. These data offer a potential explanation for the diverse responses of OC to the same ligand.

Materials and Methods

Cell cultures

Several osteoclast sources were utilized for this research. Primary human osteoclasts were used for immunofluorescence because the antibodies used to detect β_3 in all conformations (AP3 and 1A2) and in the high affinity conformation (AP5 and WOW1) recognize only human β_3 integrin. Murine osteoclasts and/or the human osteoclast-like cell line GCT 23 were used for bone resorption, migration and flow cytometry experiments.

Primary osteoclasts

Human osteoclasts were obtained as described previously by mechanical disaggregation of small surgery specimens from osteoclastomas (Grano et al., 1996) or from human bone marrow cultures. Briefly, human bone marrow samples obtained by orthopedic surgical procedures were subjected to Ficoll-Hypaque (Ficoll, Sigma Chemical Co.; Hypaque 76, Nycomed, Princeton, NJ) gradient purification, and cells at the gradient interface were collected and cultured overnight in the presence of 20 ng/ml of recombinant human M-CSF (R&D Systems Inc., Minneapolis, MN). Non-adherent cells were plated onto coverslips or dentine slices in the presence of alpha-MEM supplemented with 20% Fetal Calf Serum (FCS, Gibco Limited, Uxbridge, UK), and Vitamin D3 10^{-9} M at 37°C in a water saturated atmosphere with 5% CO_2. After 3 weeks multinucleated TRAP-positive human osteoclasts appeared.

Osteoclast-like cell line

An osteoclast-like cell line, GCT 23, cloned from a human giant cell tumor of bone has been extensively characterized for its osteoclast phenotype (Grano et al., 1994). Cells used for the experiments were all within nine and 14 passages. All the cells were maintained in Iscove's medium (IMEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin and 50 IU mycostatin at 37°C, in a water saturated atmosphere with 5% CO_2 and fed by medium replacement every 2-3 days.

Murine osteoclasts

Macrophages were isolated from bone marrow of four- to eight-week-old mice, cultured overnight in α-MEM containing 10% heat-inactivated FBS and subjected to Ficoll-Hypaque (Ficoll, Sigma Chemical Co.; Hypaque 76, Nycomed) gradient purification. Cells at the gradient interface were collected and cultured in the presence of 10 ng/ml recombinant M-CSF (R&D Systems Inc.) and 100 ng/ml RANKL for 3-4 days.

Antibodies and proteins

Recombinant osteopontin (OPN) was the kind gift of K. O. Johanson (SmithKline Beecham, King of Prussia, PA). Laminin from human placenta (LN-2) was from Sigma Chemical Co (St Louis, MO). All the antibodies were protein-A purified IgGs from ascites fluid. LM609 is a blocking anti $\alpha_v\beta_3$ antibody (Cheresh, 1991) and was kindly supplied by David Cheresh (The Scripps Research Institute, La Jolla, CA); WOW1, a monoclonal antibody which specifically binds the activated conformation of $\alpha_v\beta_3$, was generously provided by S. Shattil (The Scripps Research Institute, La Jolla, CA); AP3, a monoclonal antibody which recognizes all forms of β_3, and AP5, a monoclonal antibody against the activated conformation of β_3, have been extensively characterized (Honda et al., 1995); 1A2, an mAb against human β_3 was a gift of S. Blystone, Department of Cell and Developmental Biology, State University of New York (SUNY), Upstate Medical University at Syracuse, NY.

Monoclonal antibodies against the α_v integrin subunit were purchased from Telios Pharmaceuticals, Inc (San Diego, CA). Human recombinant macrophage colony stimulating factor (M-CSF) was from Genetics Institute (Cambridge, MA). Affinity-purified HGF was a kind gift of P. M. Comoglio (IRCC, University of Torino, Italy).

Immunofluorescence

Freshly isolated human osteoclasts were plated onto bone slices, hydroxyapatite-coated coverslips (Osteologic, Millenium Biologix Inc., Kingston, Ontario, Canada) or glass coverslips in α-MEM supplemented with 20% FHS (fetal horse serum) at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hours, cells were incubated with HGF or M-CSF in IMEM supplemented with 0.5% BSA for 30 minutes at 37°C. Control cells were incubated with α-MEM + 0.5% BSA alone. After incubation, cells were washed three times with PBS and fixed in 3% paraformaldehyde, 2% sucrose in PBS pH 7.6 for 10 minutes at room temperature. After rinsing in PBS, cells were made permeable to antibodies by soaking coverslips for 3 minutes at 0°C in Hepes-Triton X-100 buffer (20 mmol/l Hepes pH 7.4, 300 mmol/l sucrose, 50 mmol/l NaCl, 3 mmol/l $MgCl_2$ and 0.5% Triton X-100).

For indirect immunofluorescence, the primary antibody was layered on fixed and permeabilized cells and incubated in a humidified chamber for 45 minutes at 37°C. The distribution of AP5 and WOW1 were also evaluated in live cells using Abs incubated for 45 minutes at 4°C to prevent receptor internalization. Cells were then fixed and permeabilized as described previously. After rinsing in PBS (pH 7.6), coverslips were incubated with the appropriate Cyanine-3 conjugated secondary Ab (Cy-3, Chemicon, CA) and with 10 μg/ml fluorescein-labeled phalloidin (F-PHD, Sigma) for 45 minutes at 37°C. Stained coverslips were than mounted in 20% Mowiol 4-88 (Calbiochem-Novabiochem Corporation, La Jolla, CA). Observations were performed by epifluorescence in a Zeiss axioplan microscope.

Osteoclasts plated onto bone slices were viewed with a confocal microscope in the M.I.A. laboratory at DIBIT San Raffaele in Milan, Italy.

Flow cytometry

GCT23 cells were removed from growth plates by a brief treatment with Trypsin/EDTA (Sigma) and washed three times in a calcium-free buffer based on Hanks Balanced Salt Solution (HBSS). Pretreated cells were incubated with HGF or M-CSF in IMEM supplemented with 0.5% BSA for 30 minutes at 37°C; control cells were incubated with IMEM alone. After incubation with or without the growth factors, cells were washed twice with HBSS buffer supplemented with calcium and magnesium and incubated with the mAb AP5 (50 μg/ml) against the activated β_3 integrin subunit in high calcium buffer for 45 minutes on ice. A positive control was performed by incubating cells with AP5 in HBSS calcium-free buffer. Cells were then rinsed and treated with FITC-conjugated goat-anti-mouse serum (Sigma) for 30 minutes on ice. Finally, cells were suspended in 0.5 ml of HBSS buffer in the presence of propidium iodide (500 ng/ml, Sigma) added immediately prior to analysis on a Becton-Dickinson FACScan. Appropriate forward- and side-scatter gates were set for these cells, and only those cells excluding propidium iodide were included in the analysis of FITC fluorescence.

In vitro bone resorption assay

Murine osteoclasts from bone marrow macrophages were generated on whale dentin slices or on BD BioCoat™ Osteologic™ Bone Cell Culture System (Millenium Biologix) as described above. After 3 days in culture in the presence of 25 ng/ml M-CSF and 100 ng/ml RANKL, the culture media was changed and HGF (50 ng/ml), M-CSF (100 ng/ml) or vehicle alone were added for the next 48 hours. Cells on dentine slices were fixed in 4% PFA for 10 minutes at room temperature and TRAP stained. Resorptive pits were analyzed by light microscopy and pictures were taken with a 16× objective.

At the same time point, osteoclasts plated onto dentine slices were removed by a brief treatment with domestic bleach, and resorptive areas were analyzed by light microscopy.

Migration assay

Haptotactic migration assays were performed in transwell plates with an 8 μm pore size (Costar, Cambridge, MA) as described previously (Pelletier et al., 1996). Briefly, the lower side of the membrane was coated with OPN or LN at the concentration of 10 μg/ml for 2 hours at room temperature. Both sides of the membrane were blocked with 2% heat-denatured BSA for 1 hour. Cells were preincubated as described above, and 10^4 cells, diluted in IMEM supplemented with 0.5% FCS, were added per well. Following incubation at the indicated times at 37°C, cells were stained with ADiff-Quick@ (Dade Diagnostics, Aquada, PR). The cells attached to the top surface of the membrane were removed with cotton swabs. Cells that migrated to the lower side were viewed at 300× magnification, and the number of cells per field was counted. Results represent the averages from 15 fields±s.e. of a representative experiment. For inhibitory experiments, cells, after pre-incubation with AP5, HGF or M-CSF, were incubated for an additional 30 minutes at 37°C with the function-blocking mAb LM609 or the PI3-kinase inhibitor Wortmannin (Sigma).

Statistics

Statistical analysis was performed by unpaired Student's *t*-test. The results are expressed as average±s.e.

Results

$\alpha_v\beta_3$ activation by HGF and M-CSF in human osteoclast-like cells

We have demonstrated that $\alpha_v\beta_3$ exists in human osteoclast-like cells in two conformational states (Faccio et al., 1998), with the 'activated' state, recognized by the monoclonal Ab AP5, stimulating cell adhesion and migration toward $\alpha_v\beta_3$

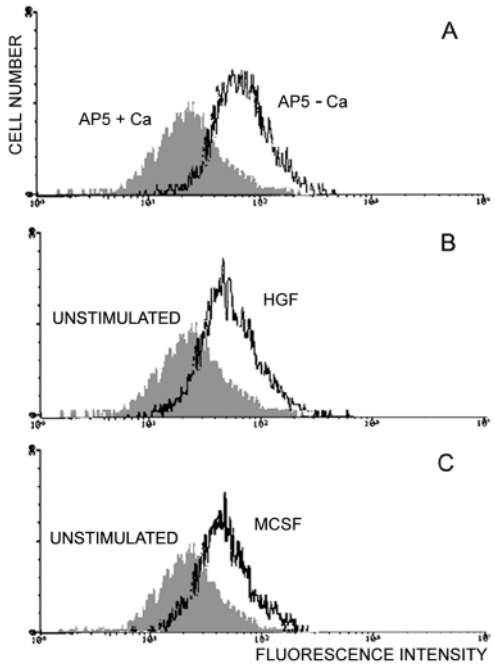

Fig. 1. Growth factor pre-treatment increases cell surface expression of 'activated' $\alpha_v\beta_3$. (A) Unstimulated human osteoclast-like cells were stained with the anti-β_3 mAb AP5 in the presence of 1.3 mM calcium (AP5+Ca) or in calcium-free buffer (AP5–Ca). In the presence of divalent cations, AP5 recognizes the fraction of the $\alpha_v\beta_3$ integrin in the 'activated' conformation. Incubation of cells with AP5 in calcium-free buffer shifts all the receptor into the 'activated' conformation. (B,C) Pre-incubation with HGF (B) or with M-CSF (C) increases the binding of AP5 in 1.3 mM calcium buffer, shifting most of the integrin into the 'activated' conformation. Mean fluorescence intensity is indicated on the abscissa; the number of fluorescence-positive cells is depicted on the ordinate.

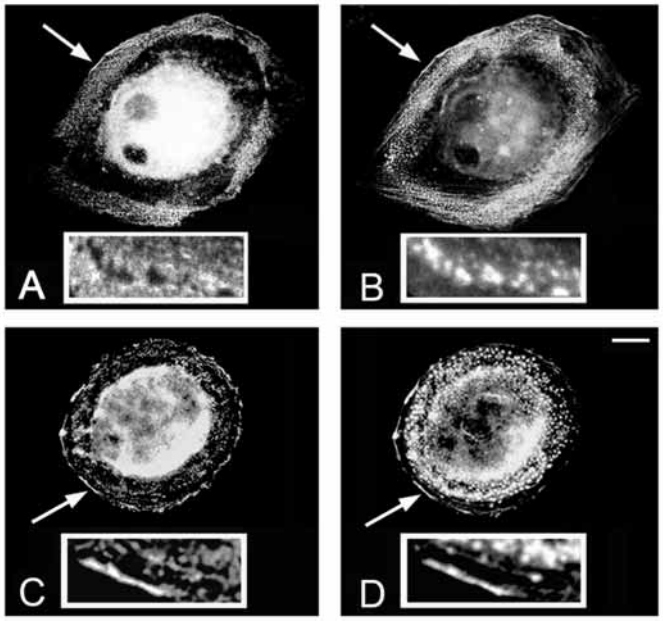

Fig. 2. Immunolocalization of total and 'activated' $\alpha_v\beta_3$ integrin in non-resorbing human osteoclasts. Primary human OCs were plated on coverslips in the presence of 10% FBS and immunostained for total $\alpha_v\beta_3$ with mAb AP3 (A) or with AP5 (C), which binds specifically to the 'activated' conformation. Cells were co-stained with F-PHD for actin (B,D). Total $\alpha_v\beta_3$ is found in rosette-like structures clustered around actin cores (Fig. 2A,B, insert) and in the motile parts of the plasma membrane (arrow). The 'activated' conformation of the integrin detected by AP5 (C) is clustered in the motile region of the plasma membrane, corresponding to ruffles and lamellipodia. At the periphery, as indicated by the arrow (Fig. 2C, insert), the integrin appears as a continuous line that colocalizes with the actin fibers (Fig. 2D, insert). Bar, 10 μm.

ligands. In transformed cells, growth factors promote cell migration by increasing integrin affinity (Trusolino et al., 2000). Thus, our first exercise was to determine whether cytokines or growth factors could regulate $\alpha_v\beta_3$ integrin conformations in human osteoclast-like cell line GCT 23. We turned to HGF and M-CSF because of their impact on OC activity and differentiation (Felix et al., 1994; Fuller et al., 1993; Grano et al., 1996). We used human osteoclast-like cell line and not primary OCs in order to obtain a large number of cells. Flow cytometric analysis of human osteoclast-like cells with AP5 was used to examine the effects of HGF and M-CSF on $\alpha_v\beta_3$ conformations. Since in the presence of low calcium concentrations AP5 induces the activation of all $\alpha_v\beta_3$ (Faccio et al., 1998), we used this condition (AP5 –Ca) to detect total β_3 (Fig. 1A). By contrast, in high calcium, buffer AP5 recognizes only 'activated' $\alpha_v\beta_3$, and we used this condition (AP5+Ca) to determine the degree of $\alpha_v\beta_3$ activation in unstimulated cells (Fig. 1A). Pre-incubation of human osteoclast-like cells with HGF (5 ng/ml) (Fig. 1B) or M-CSF (0.5 nM) (Fig. 1C) for 30 minutes at 37°C, prompts increased binding of AP5 in high calcium buffer, indicating that both HGF and M-CSF can induce the 'activated' $\alpha_v\beta_3$ conformation. Because phosphatidyl inositol 3-kinase (PI 3-kinase) has been implicated in integrin activation in leukocytes and platelets (Shimizu et al., 1995; Kovacsovics et al., 1995; Zell et al., 1996; Zhang et al., 1996), we analyzed its role in growth-factor-mediated $\alpha_v\beta_3$ activation in OCs. Addition of wortmannin, the PI 3-kinase inhibitor, to the pre-incubation medium prevents the integrin-activating effect of HGF or M-CSF (data not shown), indicating that PI 3-kinase activity is required for $\alpha_v\beta_3$ activation.

$\alpha_v\beta_3$ conformations and localization in human osteoclasts

Having established the presence of the $\alpha_v\beta_3$ integrin in different conformational states in human osteoclast-like cells GCT 23, we analyzed their expression in human osteoclasts. Because FACS analysis detects the presence of the activated $\alpha_v\beta_3$ but not its localization, we turned to immunofluorescence as a method to detect the distribution of the two conformations of $\alpha_v\beta_3$. We used the mAb AP3, which binds to epitopes within residues 287-489 (Honda et al., 1995) and recognizes β_3 in both conformational states. In non-polarized primary OCs plated on glass, AP3 highlights $\alpha_v\beta_3$ on the OC surface in the podosomes (Fig. 2A,B) and in the motile parts of the plasma membrane, called lamellipodia (Fig. 2A,B arrow). At higher magnification (Fig. 2A, insert), AP3 demonstrates that $\alpha_v\beta_3$ is organized in rosette-like structures that surround a core of actin filaments in the podosomes (Fig. 2B, insert). We have also seen

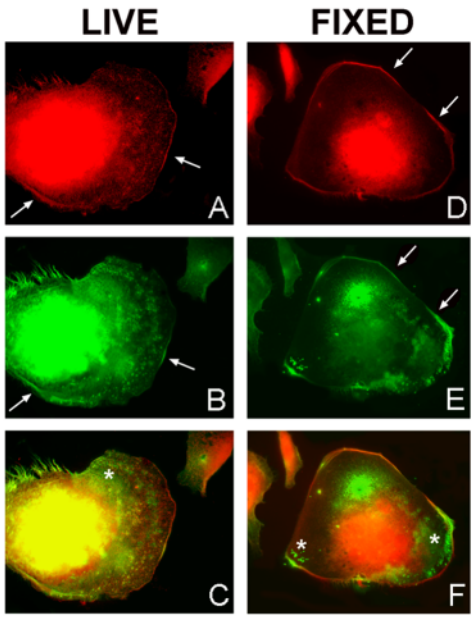

Fig. 3. Localization of 'activated' $\alpha_v\beta_3$ integrin in live and fixed cells. Human OCs were stained before (A-C) or after (D-F) fixation with the monoclonal activating Ab WOW-1 (red), which binds to the 'activated' form of $\alpha_v\beta_3$, and costains with fluorescein-tagged phalloidin to detect the actin organization (green). The 'activated' form of $\alpha_v\beta_3$ (A,D, arrows) is specifically organized at the cell edge where it colocalized with cortical actin (B,E, arrows). Clusters of podosomes are also evident in both fixed and live cells, but in both cases the 'activated' form of $\alpha_v\beta_3$ is not organized around the podosomal core of actin filaments as shown in the overlay (C,F, asterisk)

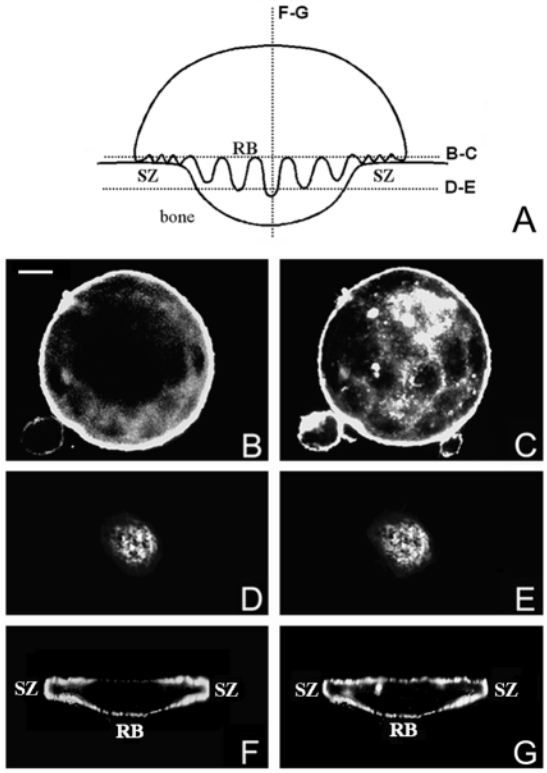

Fig. 4. Localization of total $\alpha_v\beta_3$ in resorbing OC. A resorbing OC is schematically represented in A. It appears as a polarized cell, attached to the bone through the sealing zone (SZ), that forms a specific membrane domain, the ruffled border (RB) involved in the dissolution of the bone matrix. Primary human OCs were plated onto bone slices in the presence of 10% FBS, and stained by double immunofluorescence with the anti-β_3 mAb AP3 followed by cyanine-3-conjugated rabbit anti-mouse IgG (B,D,F) and by fluorescein-tagged phalloidin (C,E,G). Microscopic images show the three different axial sections indicated in A: (B-C) at the level of the SZ, where $\alpha_v\beta_3$ (B) appears as a circular line at the cell periphery that perfectly colocalizes with the actin ring (C); (D-E) in RB, $\alpha_v\beta_3$ and the actin show a punctuate distribution; (F-G) an optical yz-plane confirms the distribution of the integrin and of the actin in the SZ and in the RB. Bar, 5 μm.

this particular organization of the β_3 integrin around the podosomal actin core in avian OCs (Marchisio et al., 1984). Similar results were obtained using the mAb 1A2, which binds to an external domain of the β_3 subunit (not shown), and using an anti-α_v mAb, which exhibited a complete overlap with the β_3 distribution (data not shown). By contrast, the 'activated' conformation of the β_3 chain, which is recognized by AP5, was primarily detected at the edge of the cell, where it colocalized with a meshwork of actin filaments in lamellipodia and membrane ruffles (Fig. 2C,D, insert) but was mostly absent from the podosomes. To confirm that the different distribution of the 'activated' receptor was not caused by an artifact dependent on fixation procedures or to non-specific binding of AP5, cells were stained with a second Ab that recognizes 'activated' $\alpha_v\beta_3$, WOW1, before and after fixation. In Fig. 3, WOW1 staining of human osteoclasts shows a complete concordance with the AP5 distribution. In both live and fixed cells, the 'activated' receptor colocalized at the cell edge with the cortical actin (A-B and D-E arrows), and, in contrast to the 'basal' receptor, was absent from the podosomes (C,F, asterisk). On the basis of these findings, we performed all the following immunofluorescence staining experiments on fixed cells to avoid possible modifications, such as receptor internalization, during the Ab incubation.

As shown in Fig. 4A, resorbing OCs are polarized cells that adhere to the bone through the sealing zone (SZ) and form a specific membrane domain, the ruffled border (RB), which is involved in the dissolution of mineralized bone matrix. Having shown that the two different conformational states of the integrin coexist in non-resorbing OCs plated on coverslips, we examined the distribution of the two conformational states of $\alpha_v\beta_3$ during bone resorption. Human OCs were plated onto bone slices, stained with AP3 or AP5 mAbs and observed by confocal microscopy. In the cross-section from the sealing zone, total $\alpha_v\beta_3$, as visualized with AP3 (Fig. 4B), appeared as a circle at the cell periphery (SZ) that perfectly colocalized with the actin ring (Fig. 4C), suggesting that the receptor participates in recognition of bone matrix. The cross-section shown in panels D and E revealed the $\alpha_v\beta_3$ distribution in the ruffled border (Fig. 4D) where actin filaments were also detected (Fig. 4E). The same cell has been analyzed through an optical yz plane (F-G). In this cross section, the overlap of $\alpha_v\beta_3$ and actin in the sealing zone (SZ), as well as their localization in the ruffled border (RB), was evident. By contrast, the receptor in the 'activated' conformation, detected by AP5, only partially colocalized in a punctuate pattern with

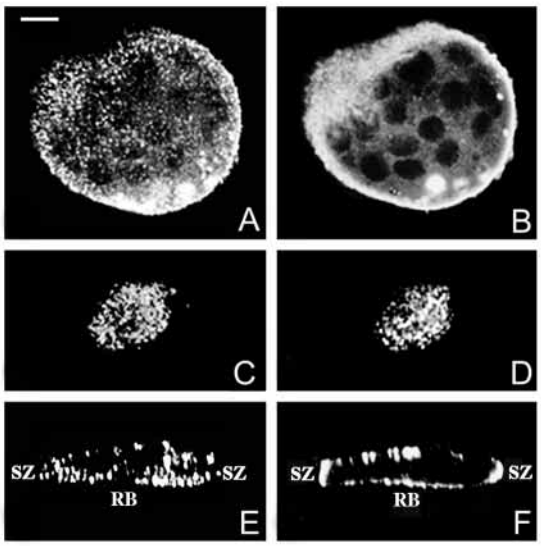

Fig. 5. Localization of 'activated' $\alpha_v\beta_3$ in resorbing OC. Primary human OCs were plated onto bone slices in the presence of 10% FBS and stained by double immunofluorescence with the anti-β_3 mAb AP5, which recognizes only the 'activated' integrin conformation, followed by cyanine-3-conjugated rabbit anti-mouse IgG (A,C,E) and by fluorescein-tagged phalloidin (B,D,F). Microscopic images show the three different axial sections indicated in Fig. 3A: at the level of the sealing zone (SZ), the 'activated' β_3 (A) only partially colocalizes with the actin ring (B) and displays a discrete pattern. In the ruffled border area (RB) the 'activated' β_3 (C) is very abundant with a punctuate distribution similar to that of the actin microfilaments of D. In the optical yz-plane, note the presence of the actin in the sealing zone SZ (F) as a line at the edge of the cell that only partially colocalizes with the integrin detected by AP5 (E). By contrast, the 'activated' receptor and the actin are both present in the ruffled border area RB. Bar, 5 μm.

actin in the sealing zone (Fig. 5A,B), a distribution confirmed by analysis of the same cell through the yz plane (Fig. 5E,F in SZ). The 'activated' integrin was strongly detected in the ruffled border (Fig. 5C,D; Fig. 5E,F in RB) with a similar distribution to the AP3 staining. On the basis of these observations we can conclude that the 'activated' form is present mainly at the edge of the ruffled border, whereas the 'basal' conformation is also present in the sealing zone. These findings suggest that the two conformational states of $\alpha_v\beta_3$ are differently distributed, indicating that they may exert distinct effects on OC activity.

HGF and M-CSF induce changes in $\alpha_v\beta_3$ distribution in non-resorbing OCs

We next determined whether both 'basal' and 'activated' conformation of $\alpha_v\beta_3$ could be modulated by growth factor treatment. Human OCs plated on coverslips were treated with HGF, M-CSF or vehicle alone and immunostained with the mAb AP3, which recognizes both $\alpha_v\beta_3$ conformational states. In untreated cells, $\alpha_v\beta_3$ was organized in rosette-like structures (Fig. 6A-C), as described previously (Fig. 2A,B). By contrast, pre-incubation with HGF or M-CSF for 30 minutes at 37°C alters the distribution of $\alpha_v\beta_3$, shifting the integrin away from podosomes to the protrusive edges of the plasma membrane (Fig. 6D-F and 6G-J). The distribution of the 'activated' $\alpha_v\beta_3$ detected by AP5 and WOW-1 mirrors immunostaining with AP3 (not shown), indicating that the majority of the integrin has been activated by the growth factors.

Flow-cytometric analysis revealed that PI 3-kinase is required for growth-factor-mediated $\alpha_v\beta_3$ activation in osteoclast-like cells (not shown). In mature OCs, PI 3-kinase associate with $\alpha_v\beta_3$ (Hruska et al., 1995; Lakkakorpi et al., 1997). In our system, adding the PI 3-kinase inhibitor wortmannin before growth factor stimulation blunted the $\alpha_v\beta_3$ redistribution (Fig. 6K-M). The receptor was still organized in podosomes and failed to localize at the cell edge. These results suggest that PI 3-kinase is important for the activation and

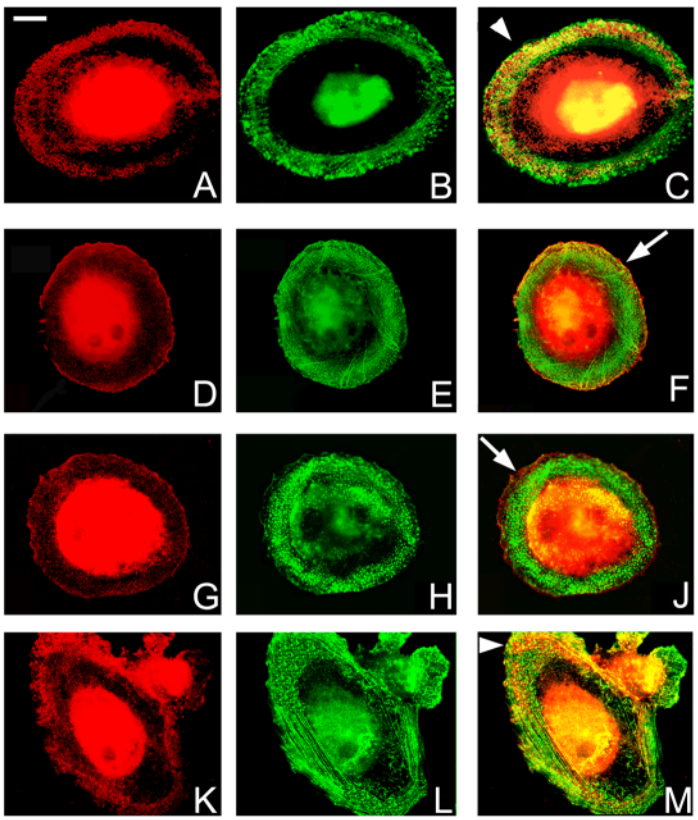

Fig. 6. Pre-treatment with HGF and M-CSF in resting osteoclasts promotes $\alpha_v\beta_3$ redistribution in membrane ruffles and lamellipodia. Primary human osteoclasts plated onto coverslips were preincubated with vehicle alone (A-C), HGF (5 ng/ml) (D-F) or M-CSF (0.5 nM) (G-J) and immunostained for total $\alpha_v\beta_3$ with AP3 and co-stained with FITC-phalloidin. Pseudocolor merged images from $\alpha_v\beta_3$ staining in red and actin in green have been examined. The typical distribution of $\alpha_v\beta_3$ in rosette-like structures around a core of actin filaments in the podosomes, detected by AP3 in vehicle-treated osteoclasts (A-C, arrowhead), has been replaced by a peripheral distribution in membrane ruffles and lamellipodia in (D-F, arrow) HGF- or (G-J, arrow) M-CSF-treated osteoclasts. Pre-incubation with wortmannin before growth factor treatment inhibited integrin mobilization to the membrane edges, and $\alpha_v\beta_3$ is still organized in rosette-like structures (K-M, arrowhead). Phalloidin staining (B,E,H,L) shows that the podosomes in all treated cells are still well organized. Wortmannin-treated cells also show filaments of cortical actin (L). Bar, 10 μm.

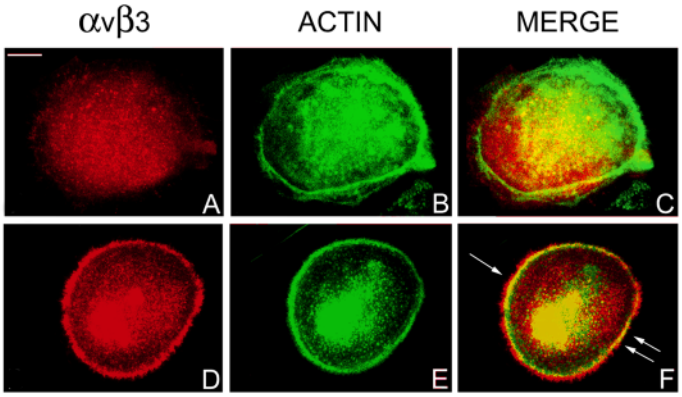

Fig. 7. Redistribution of $\alpha_v\beta_3$ occurs in resorbing human OCs pre-incubated with HGF. Primary human OCs, incubated with vehicle alone (A-C) or HGF (5 ng/ml) (D-F) were plated onto hydroxyapatite-coated coverslips (BD BioCoat™ Osteologic™) and examined by immunofluorescence, followed by staining with AP5 (A,D) and FITC phalloidin (B,E). Without exogenous growth factors, the activated $\alpha_v\beta_3$ is distributed with a punctuate pattern on the cell surface (A,B). A pseudocolor merged image (C) from $\alpha_v\beta_3$ staining in red (A) and actin in green (B) fails to reveal colocalization of the integrin with the actin ring. HGF treatment induces a change of conformation of $\alpha_v\beta_3$ that is found mostly in fillopodia-like extensions (D). The pseudocolor merged image (F) reveals $\alpha_v\beta_3$ (red) mainly localized in small protrusions at the cell periphery, external to the actin ring (green) in the sealing zone (single arrow). A partial colocalization between the integrin and the actin ring is seen in yellow (double arrows). Bar, 5 μm.

redistribution of $\alpha_v\beta_3$ under growth factor stimulation. Thus, HGF and M-CSF mobilize $\alpha_v\beta_3$ from the cytoskeleton, inducing its relocalization in a PI-3-kinase-dependent manner.

Growth factors change the distribution of activated $\alpha_v\beta_3$ in resorbing OCs

Given the changes in $\alpha_v\beta_3$ conformation in non-resorbing cells in response to HGF and M-CSF, we next turned to the effects of these cytokines on resorbing OCs. Human OCs were plated onto hydroxyapatite-coated coverslips (BD BioCoat™ Osteologic™), which efficiently substitute for bone slices. Although this inorganic substrate does not itself contain the organic matrix components found in bone, OC resorptive activity does depend on the presence of $\alpha_v\beta_3$ integrin (R.F. and D. Novack, unpublished). The necessary organic components may be secreted by the osteoclastic cells and/or contained in the serum-supplemented media. Mature human osteoclasts were treated with growth factors or vehicle and immunostained with AP5. As we saw in OCs on bone (Fig. 5), high affinity $\alpha_v\beta_3$ is confined mainly to the ruffled border and is distributed with a punctuate pattern in the sealing zone in the absence of exogenous growth factors (Fig. 7A,C). Treatment with HGF (Fig. 7D,F) or M-CSF (not shown) for 30 minutes modified the integrin conformation, including the fraction in the sealing zone that appeared brightly stained by AP5 (Fig. 7D). However, the 'activated' integrin only partially colocalized (yellow) with the actin ring, as demonstrated by the merge between $\alpha_v\beta_3$ staining in red and actin in green (Fig. 7F). The 'activated' integrin after growth factor treatment was also detected in small protrusions at the cell periphery external to the actin ring. Longer incubation (8 hours) yielded dramatic morphological changes in resorbing OCs. The cells started to migrate and the integrin was eventually found in more organized motile areas of the plasma membrane, such as lamellipodia and fillopodia (not shown).

HGF and M-CSF increase bone resorption

To assess the functional implications of HGF- and M-CSF-induced alterations in $\alpha_v\beta_3$ activation, we determined the effect of the growth factors on osteoclastic bone resorption. Murine bone marrow macrophages were plated onto whale dentine slices or hydroxyapatite-coated slides and cultured in the presence of 100 ng/ml RANKL and 25 ng/ml M-CSF. After 3 days, the presence of mature OCs was confirmed by TRAP staining of parallel cultures. At this time, the culture media was replaced with a fresh one containing 50 ng/ml of HGF or 100 ng/ml M-CSF. After an additional 48 hours, cells on dentine slices were fixed and TRAP stained, and the bone resorptive lacunae were analyzed by light microscopy (Fig. 8A-C). Compared with untreated cells (A), HGF (B) and M-CSF (C) treatment increased the pit number 2.6-and 2.3-fold, respectively. To evaluate the resorbed area, hydroxyapatite-coated slides were treated with bleach to remove cells, and the cleared area was quantified. Unstimulated OCs resorbed 30% of the total area (Fig. 8D), whereas HGF (E) and M-CSF (F) increased the resorptive area to 65% and 58%, respectively.

Activated $\alpha_v\beta_3$ increases osteoclast migration to OPN in a PI-3-kinase-dependent manner

Normal resorptive function of OCs depends in part upon their ability to migrate over the bone surface to initiate new sites of bone resorption. Having shown an increased bone resorption in OCs treated with HGF and M-CSF, we determined whether it was correlated with an increased motility. Haptotactic migration of human osteoclast-like cells was measured in a transwell assay in which the bottom of the membrane had been coated with OPN (10 μg/ml). Cells, which had migrated through the membrane to the OPN-coated surface, were counted. Pretreatment with either growth factor increased human osteoclast-like cell migration to levels comparable to those pre-treated with AP5 in low calcium, which we had previously established promotes motility (Faccio et al., 1998) (Fig. 9A). This increase in cell migration was inhibited by the $\alpha_v\beta_3$ blocking mAb LM609 (Fig. 9A). In contrast to OPN, human osteoclast-like cell migration toward laminin (LN), a substrate not recognized by $\alpha_v\beta_3$, was not affected by HGF or M-CSF pretreatment nor inhibited by LM609 (Fig. 9B), indicating a specific role for the two growth factors in $\alpha_v\beta_3$-dependent migration.

Having established that the PI-3-kinase inhibitor wortmannin blocks growth-factor-mediated redistribution of $\alpha_v\beta_3$ (Fig. 6K-M), we next examined whether this treatment would also affect growth-factor-mediated OC migration. After

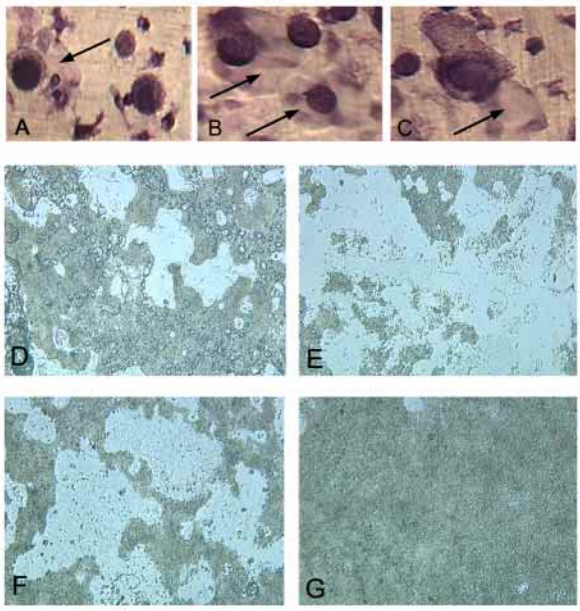

Fig. 8. HGF and M-CSF enhance bone resorption. Murine bone marrow macrophages were cultured onto dentine slices (A-C) or millennium slides (D-F) for 3 days in the presence of RANKL (100 ng/ml) and M-CSF (10 ng/ml). HGF (50 ng/ml), M-CSF (100 ng/ml) or vehicle alone were added for the next 48 hours and bone resorptive activity was analyzed. (A-C) After indicated treatments, cells plated onto dentine slices were fixed, TRAP stained and subjected to light microscopy. Pits, indicated by arrows, increased in number and area in (B) HGF- and (C) M-CSF-treated OCs compared with the control (A). (D-G) Similar experiments were performed onto millennium slides. At the indicated time, osteoclasts were removed by brief treatment with bleach, and resorptive areas were analyzed by light microscopy (identified as the light blue regions). HGF (E) and M-CSF (F) increased erosion areas by 65% and 58%, respectively, compared with vehicle-treated OCs (D). G shows a control well with no cells. All images are at the same magnification.

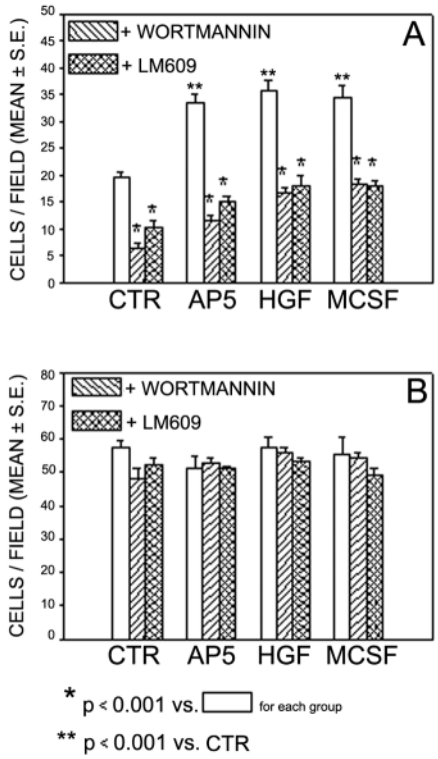

Fig. 9. Pre-incubation with HGF and M-CSF induces an increase in cell motility toward OPN, in a $\alpha_v\beta_3$- and a PI-3-kinase-dependent manner. (A) Migration towards OPN. Human osteoclast-like cells (open bars) preincubated with AP5 in buffer without calcium, HGF (5 ng/ml) or M-CSF (0.5 nM) are allowed to migrate for 6 hours toward OPN (10 μg/ml), coated on the bottom membrane of a transwell plate (8 μm pore size). All three treatments significantly increased (P<0.001) migration toward OPN compared with vehicle alone (CTR) (open bars). Cell migration was inhibited by the $\alpha_v\beta_3$-blocking mAb LM609 (hatched bars), indicating specific involvement of the integrin $\alpha_v\beta_3$, and by the PI 3-kinase inhibitor wortmannin (cross-hatched bars). (B) Migration toward LN. Migration of human osteoclast-like cells toward LN is not enhanced by growth factor or AP5 pre-treatment (open bars) nor decreased by LM609 (hatched bars) or wortmannin (cross-hatched bars). In all experiments cells that migrated were viewed at 300× magnification, and the number of cells per field was counted.

pre-incubation with HGF, M-CSF or AP5 in the presence of low calcium, as a control for integrin activation, wortmannin (200 nM) was added to the medium and cells were allowed to migrate toward OPN or LN. As seen in Fig. 9A, wortmannin substantially reduced cell migration towards OPN to levels comparable with anti-$\alpha_v\beta_3$ mAb-mediated inhibition. The inhibitor was completely ineffective in the altering migration toward LN (Fig. 9B). This result and the previous morphologic observations suggest that PI 3-kinase activity is required for growth-factor-induced cell migration via recruitment of the 'activated' $\alpha_v\beta_3$ to the motility related sites.

Discussion

The activity of integrins in several cell types is tightly controlled, and adhesion and/or motility can depend upon a shift from a 'basal' to a 'activated' state of the receptor. The leukocyte-specific integrin LFA-1 is expressed at the cell surface in an inactive state but can rapidly be converted into an active state, which leads to enhanced ICAM-1 binding during transmigration across the vasculature (vanKooyk et al., 2000). Similarly, the $\alpha_{IIb}\beta_3$ integrin is expressed in a 'basal' state in circulating platelets and is converted into an 'activated' state enabling it to bind to fibrinogen during clot formation. Previously we showed that $\alpha_v\beta_3$ exists in different functional states in osteoclast-like cells (Faccio et al., 1998). The 'activated' conformation increases affinity to the ligand, leading to a higher efficiency in migration toward osteopontin. Two distinct mechanisms have been put forward for the integrin activation. The first is that there is an increase in the intrinsic affinity of the integrin to its ligand owing to conformational change, and the second is that the avidity of the receptor increases as a result of receptor clustering at the cell surface. Changes in integrin affinity (interaction of a single integrin with its ligand) can be detected by antibodies that identify activation-dependent neo-epitopes.

In the current study we utilized two different antibodies, WOW-1 and AP5, to detect the 'activated' $\alpha_v\beta_3$ integrin. WOW-1, a monovalent Ab, binds only to unoccupied, activated integrins and is insensitive to changes in integrin clustering.

AP5, in contrast, binds to the N-terminal domain of the β_3 subunit stabilizing and/or inducing its active conformation. Certain cations (e.g. Ca^{2+}) can compete with AP5 in binding to the receptor when in the inactive conformation. The advantage of using AP5 is that it can be used to detect the fraction of integrin that is 'activated' (at high calcium) and the fraction of 'activatable' integrin (at low calcium).

The crystal structure of the $\alpha_v\beta_3$ integrin has been solved recently by two different groups (Beglova et al., 2002; Xiong et al., 2001). The C-termini of the α_v and β_3 extracellular domains are very close together in the $\alpha_v\beta_3$ structure (Xiong et al., 2001), mimicking the close association in the juxtamembrane region that maintains integrin in the inactive state. In this genuflected conformation, the activation epitopes are also masked. Integrin activation has been correlated with flexibility at the 'genu'. Therefore an extended form, which is commonly seen in electron micrographs of integrins, including integrin simultaneously bound to ligand and activating antibody, exposes a much broader surface, including several activating epitopes. AP5, a monoclonal activating antibody used in the current study, binds to the first six amino acids in the N-terminal end of the β_3 subunit, which are localized in the PSI (plexin-semaphorin integrin) domain (Honda et al., 1995). Unfortunately the structure of this terminal region, localized in the genu motif, has not been solved. It is possible that the binding of AP5 is completely masked in the genuflected conformation (inactive) but is exposed in the extended configuration (active), as demonstrated for other activating antibodies (Beglova et al., 2002).

In the current paper we have identified the localization of two different $\alpha_v\beta_3$ conformations in resting and resorbing human osteoclasts derived from several sources (bone marrow culture, osteoclastoma and a giant cell tumor of bone-derived cell line). We have utilized two antibodies to detect the 'activated' $\alpha_v\beta_3$ conformation, AP5 and WOW-1, which recognize different epitopes localized in the β_3 subunit, and both have shown same pattern of distribution in both live and fixed cells. Both antibodies specifically bind to the fraction of integrin organized at the cell edge, where it colocalizes with filaments of cortical actin. By contrast, the antibodies to total $\alpha_v\beta_3$, AP3 and 1A2 identify an additional pool of 'basal' integrin in the podosomes and in the sealing zone, where it is associated with the actin ring. It is not clear why the 'basal' and not 'activated' integrin is found in the sealing zone where there is very tight membrane apposition to the bone surface, as observed in electron micrographs of resorbing OC. It is possible that the extended conformation of activated integrin, seen in the crystal structure, is too large to fit in this space. The concentration of integrin in the sealing zone is great, so even with 'non-activated' integrin, the avidity is high at this site.

Even in the absence of growth factors, the integrin exists in both 'basal' and 'activated' forms. Growth factor treatment, which promotes cell motility, induces both redistribution and activation of the $\alpha_v\beta_3$ integrin. The integrin moves from the podosomes, in which the receptor is found in a 'basal' conformation, to the new forming leading edges, where the receptor is in an 'activated' state. These changes in the integrin are accompanied by functional changes in the OC (increased migration and resorption) and suggest that the two pools of integrin serve different functions.

Evidence that integrins and growth factor receptors share common signaling pathways (Clark and Brugge, 1995), suggests that signals induced by growth factors could be responsible for integrin activation (Trusolino et al., 2000; Trusolino et al., 1998). We studied the effects of HGF and M-CSF because of their ability to influence osteoclast differentiation, survival and activity. Flow cytometric analysis confirms the ability of the two growth factors to induce integrin activation as measured by binding of the mAb AP5 in the presence of divalent cations.

Following growth factor exposure, cytoskeletal rearrangements occur, allowing lateral movement of the integrin and leading to $\alpha_v\beta_3$ translocation to the motile regions of the plasma membrane. In resorbing OCs, growth factor treatment causes dynamic reorganization of the 'activated' integrin into the marginal area of the cell around the clear zone in fillopodia-like structures. Consequently, this integrin reorganization supports the translocation of OCs to new sites of the bone surface to be resorbed and explains the increased bone resorption found in the presence of HGF and M-CSF (Fig. 8). Migration assays confirm the pivotal role of the activated receptor during this process. HGF or M-CSF pre-incubation induces a $\alpha_v\beta_3$-dependent and -specific increase in osteoclast motility toward osteopontin but has no effect on cell migration toward LN. Nakamura et al. show that M-CSF treatment causes recruitment of the $\alpha_v\beta_3$ integrin to adhesion contacts and induces stable association with PLC-γ, PI 3-kinase and Pyk2 (Nakamura et al., 2001). In that system the authors did not address the question of whether the 'activated' $\alpha_v\beta_3$ is mediating the recruitment of this molecular complex. In our study we found that the growth factor treatment induces activation of most $\alpha_v\beta_3$ expressed in OCs and that PI 3-kinase is required for this effect.

PI 3-kinase has been implicated in integrin activation in leukocytes and platelets (Shimizu et al., 1995; Kovacsovics et al., 1995; Zell et al., 1996; Zhang et al., 1996). In avian OCs, $\alpha_v\beta_3$ is associated with the signaling PI 3-kinase, c-Src and FAK (Hruska et al., 1995). In that system, interaction of $\alpha_v\beta_3$ with osteopontin increases PI 3-kinase activity and association with Triton-insoluble gelsolin (Chellaiah and Hruska, 1996). In generated murine osteoclasts, PI 3-kinase translocates to the cytoskeleton upon osteoclast attachment to the bone surface (Lakkakorpi et al., 1997). We also found that the functional consequences of $\alpha_v\beta_3$ activation are PI 3-kinase dependent. First, pre-incubation with wortmannin inhibits cell migration towards the $\alpha_v\beta_3$ ligand OPN but not towards LN. Second, inhibition of PI 3-kinase activity is accompanied by the inability of HGF/M-CSF to recruit $\alpha_v\beta_3$ integrin to motility related sites. Phosphatidylinositides generated by PI 3-kinase can activate GTP-GDP exchangers for the small GTPases Rho and Rac, which, in turn, control organization of the actin cytoskeleton, inducing formation of focal adhesion, podosomes, membrane ruffles and lamellipodia (Chellaiah et al., 2000; Clark et al., 1998; Ory et al., 2000; Ridley et al., 1999). Finally, PI 3-kinase mediates M-CSF- and HGF-driven intracellular signaling. Upon HGF stimulation, the HGF receptor *c-met* exhibits tyrosine kinase activity, including receptor autophosphorylation, and activates a series of signal transducing proteins. Autophosphorylated *c-met* associates with PI 3-kinase in vivo and in vitro (Graziani et al., 1991; Ponzetto et al., 1993). Husson et al. also showed the formation of a multiprotein complex between c-fms, c-Cbl, PI 3-kinase and Grb2 upon M-CSF

stimulation (Husson et al., 1997). In OCs, a functional association between PI 3-kinase and c-Src has been shown during M-CSF-induced spreading (Grey et al., 2000).

In conclusion, our results indicate that the equilibrium between two integrin conformations is modified by growth factors, resulting in altered inside-out signaling and thus modulating the resorptive capability of the OCs. Although it is clear that HGF and M-CSF govern integrin activity, the precise mechanisms by which PI3-K and other signaling molecules interact with growth factors to modulate integrin conformation are still to be determined.

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