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ARTICLES

Effects of Vitaxin®, a Novel Therapeutic in Trial for Metastatic Bone Tumors, on Osteoclast Functions In Vitro

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Abstract The integrin αvβ3 mediates cell–matrix interactions. Vitaxin®, a humanized monoclonal antibody that blocks human and rabbit αvβ3 integrins, is in clinical trials for metastatic melanoma and prostate cancer. αvβ3 is the predominant integrin on osteoclasts, the cells responsible for bone resorption in health and disease. Here, we report the first investigation of Vitaxin's effects on osteoclast activity. Vitaxin (100–300 ng/ml) decreased total resorption by 50%, but did not alter resorptive activity per osteoclast. Vitaxin (300 ng/ml) decreased osteoclast numbers on plastic by 35% after 48 h. Similarly, attachment after 2 h was reduced by 30% when osteoclasts were incubated with Vitaxin (300 ng/ml) for 25 min prior to plating; however, the rate of fusion of osteoclast precursors in Vitaxin-treated and control groups was equal. Using time-lapse microscopy, we evaluated the effect of Vitaxin on osteoclast morphology and found a significant reduction in osteoclast planar area only when cells were pretreated with macrophage colony stimulating factor (M-CSF). Extracellular Ca^{2+} and M-CSF have opposite effects on αvβ3 conformation. Elevation of extracellular Ca^{2+} eliminated the inhibitory effect of Vitaxin on osteoclast attachment. In contrast, the effect of Vitaxin was enhanced in cells pretreated with M-CSF. This action of M-CSF was suppressed by the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor wortmannin, suggesting that M-CSF increases Vitaxin's inhibitory effect by inside-out activation of αvβ3. In conclusion, Vitaxin decreases resorption by impairing osteoclast attachment, without affecting osteoclast formation and multinucleation. Our data also show that Vitaxin's inhibitory effects on osteoclasts can be modulated by factors known to alter the conformation of αvβ3. J. Cell. Biochem. 102: 341–352, 2007. © 2007 Wiley-Liss, Inc.

Key words: osteoclast; integrin αvβ3; extracellular matrix; anti-cancer therapy; bone

Bone remodeling is a physiological process dependent on the balance between formation and resorption. This balance is determined by both the number and activity of osteoblasts, the bone forming cells, and osteoclasts, the bone resorbing cells [Manolagas, 2000]. Imbalance between formation and resorption leads to pathological bone loss in rheumatoid arthritis (RA) and several other bone diseases. RA is associated with, periarticular and subchondral bone loss, which contributes to joint destruction. There is still a need for RA treatments that are able to effectively relieve pain and block joint destruction [Mullan and Bresnihan, 2003].

Osteoclasts are multinucleated cells formed by fusion of hematopoietic mononuclear precursors [Teitelbaum and Ross, 2003]. They are unique in their ability to degrade both the inorganic and organic components of bone. Essential to osteoclastic bone resorption is the integrin αvβ3, although the mechanism of how αvβ3 affects osteoclast activity is not fully

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understood. $\alpha v\beta 3$ is a type I transmembrane glycoprotein receptor consisting of α and β subunits that functions as a bi-directional gateway mediating cell–matrix interactions. As the predominant integrin on the osteoclast surface [Davies et al., 1989], $\alpha v\beta 3$ is postulated to be involved in differentiation, attachment, and resorption [Hynes, 2002]. $\alpha v\beta 3$-Mediated attachment to matrix proteins such as osteopontin and vitronectin is through an RGD binding domain [Horton, 1997]. Matrix recognition and attachment of osteoclasts via $\alpha v\beta 3$ is responsible for initiation of signaling cascades activating osteoclasts [Miyamoto et al., 2000; Boyle et al., 2003]. The activated osteoclast is polarized and is characterized by two distinctive structures, the ruffled border and the sealing zone. Together, the sealing zone and the ruffled border create an isolated microenvironment of acidic pH required for mineral dissolution and matrix degradation by cathepsin K and other enzymes.

$\alpha v\beta 3$ Integrin exists in two different conformations, basal or activated [Xiong et al., 2001, 2002]. Changes in activation state correlate to ligand binding efficiency [Faccio et al., 1998; Geiger et al., 2001]. Divalent cations such as Mg^{2+} and Mn^{2+} induce activation of $\alpha v\beta 3$ integrins and enhance their ligand binding affinity. Conversely, high concentrations of Ca^{2+} shift the integrin into an inactive conformation, lowering its binding affinity [Legler et al., 2001]. Hepatocyte growth factor and macrophage colony stimulating factor (M-CSF), two growth factors involved in osteoclastogenesis, increase the number of activated receptors through inside-out activation of $\alpha v\beta 3$ [Faccio et al., 2003]. It has also been shown that M-CSF induces osteoclast spreading and cytoskeletal reorganization and consequently affects osteoclast attachment and resorptive ability. The effects of M-CSF on osteoclast structure and function are due in part to its ability to increase the affinity of $\alpha v\beta 3$. Those effects were found to be mediated through phosphatidylinositol 3-kinase (PI3-kinase) and required the presence of S752 in the $\beta 3$ cytoplasmic tail [Pilkington et al., 1998; Teti et al., 1998; Palacio and Felix, 2001; Faccio et al., 2003].

As a key step in bone resorption involves osteoclast attachment via $\alpha v\beta 3$, numerous efforts to inhibit bone resorption have been initiated using antagonists that mimic the RGD motif and competitively occupy the receptor [van der Pluijm et al., 1994]. Alternatively, blocking antibodies have been investigated because of their potential specificity for $\alpha v\beta 3$. Peptidomimetics, disintegrins, and blocking antibodies reduce resorption in vivo and in vitro, by affecting osteoclast differentiation, migration, and/or attachment [Chambers et al., 1986; Horton et al., 1991; Nakamura et al., 1996; Nakamura et al., 1999; Carron et al. 2000; Meissner et al., 2002].

In this study, we evaluated the role of $\alpha v\beta 3$ on osteoclast function using the $\alpha v\beta 3$ blocking antibody Vitaxin®. Vitaxin is a humanized monoclonal antibody that binds a conformational epitope formed by the αv and $\beta 3$ subunits (both human and rabbit) [Wu et al., 1998; Wilder, 2002]. Vitaxin is currently in clinical trials for the treatment of stage IV metastatic melanoma and androgen-independent prostate cancer [Tucker, 2006]. The outcome of phase II clinical trials showed that Vitaxin increased median survival of metastatic melanoma patients with minimal side effects. $\alpha v\beta 3$ antagonists, including Vitaxin, have been used to target both primary and metastatic tumors as the integrin is known to be expressed on tumor endothelial cells. The $\alpha v\beta 3$ integrin also affects the establishment and growth of tumors in bone through its role in initiating osteoclastic bone resorption [Gutheil et al., 2000; Patel et al., 2001; Posey et al., 2001]. Vitaxin was also considered for the treatment of RA due to its antiangiogenic properties [Wilder, 2002]. To date, there is no data available on how Vitaxin specifically affects osteoclasts and their function.

The focus of this study was to determine the effects of Vitaxin on osteoclastic resorption and the cellular mechanisms underlying these effects. We also studied if Vitaxin had different effects on specific osteoclast populations and how responses to the antibody can be modulated by factors known to affect the conformation of $\alpha v\beta 3$. Using authentic rabbit osteoclasts, we asked if Vitaxin affected osteoclast attachment and/or formation. Here, we show that Vitaxin reduces resorption by decreasing initial osteoclast attachment and cell spreading. In contrast, Vitaxin did not affect the resorptive capacity of individual osteoclasts nor did it affect the rate of osteoclast formation. Our data also suggests that the inhibitory effect of Vitaxin on osteoclasts is modulated by conformational changes in $\alpha v\beta 3$.

MATERIALS AND METHODS

Materials

Vitaxin, an anti-human αvβ3 antibody, was generously provided by Dr. Su-Yau Mao, MedImmune, Inc. (Gaithersburg, MD), sheep IgG anti-type I collagen antibody was kindly provided by Dr. J. Sodek (University of Toronto) and biotinylated donkey anti-sheep IgG was obtained from Sigma–Aldrich Ltd. (St. Louis, MO). Recombinant mouse M-CSF was purchased from Calbiochem (EMD BioSciences, Inc. San Diego, CA). α-Minimum essential medium (α-MEM) was purchased from Medstores, University of Toronto (Toronto, Ont.), fetal bovine serum (FBS), and antibiotic-antimycotic solution (100X) were obtained from Invitrogen (Carlsbad, CA). Fast red violet LB salt, naphthol AS-MX, 3,3′-diaminobenzidine tetrahydrochloride (DAB) and wortmannin were obtained from Sigma–Aldrich Ltd. (St. Louis, MO). Avidin–biotin–peroxidase complex was bought from Dimension Labs (Burlingame, CA). BioCoat™ Osteologic™ bone cell culture system (16 well), pretreated 6- and 12-well plastic Falcon™ tissue culture plates and 96-well Falcon™ microtiter plates were purchased from BD Biosciences (BD Labwares, Franklin Lakes, NJ).

Rabbit Osteoclast Isolation

Animal protocols were approved by the Animal Care Committees at the University of Toronto and the University of Western Ontario. Osteoclasts were isolated from the long bones of newly born New Zealand rabbits, as previously described [Kanehisa and Heersche, 1988; Lees et al., 2001]. Briefly, bones were cleaned and minced mechanically in 100 mm glass Petri dishes containing 10 ml of α-MEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution (supplemented media). Bone fragments were transferred into 50 ml Falcon tubes and cells were resuspended by repeated passage (30 times) through a wide-bore Pasteur pipette. The bone fragments were allowed to briefly settle and the cell suspension was transferred to another tube. An additional 6 ml of supplemented media was then added to the remaining minced bones in the Petri dish and the previous steps were repeated. This technique of cell isolation generates a mixed culture of attached osteoclasts, prefusion osteoclasts, and stromal cells. To ensure that cell counts reflected the number of osteoclasts, only multinucleated cells that stained positive for tartrate-resistant acid phosphatase (TRAP) were counted. To stain for TRAP activity, cultured cells were washed with PBS, and then fixed with 2.5% glutaraldehyde for 5 min. Staining was carried out according to the protocol described in BD Biosciences Technical Bulletin #445.

Preparation of Devitalized Cortical Bone Slices

Cortical bone slices were prepared from bovine long bone obtained from a butcher shop as described previously [Shorey et al., 2004]. Briefly, a devitalized block of bovine cortical bone was thawed. Sections of 120–150 μm thickness were obtained using a Buehler Isomet low speed saw. After polishing these sections, discs of 5 mm diameter were punched out using a cork borer. Slices were then sonicated once in distilled water for 10 min, disinfected by submersion in 70% ethanol overnight, washed in α-MEM (3× 10 min) and then incubated overnight at 37°C in supplemented medium.

Attachment Studies

Aliquots (100 μl) of cell suspension were plated in 6-well plastic plates. Osteoclasts were allowed to attach for 1 h (37°C, 5% CO_2), after which 1.5 ml of supplemented medium was added. After a further 18-h period, the cultures were washed gently with α-MEM using a wide-bore Pasteur pipette to remove non-attached cells and then incubated in supplemented media with or without Vitaxin (30, 100, and 300 ng/ml) for an additional 48 h. Cells were subsequently fixed and stained for TRAP activity.

"Initial attachment" experiments were performed by diluting the initial cell suspension 1:1 with an equal volume of supplemented media with or without Vitaxin at 4°C for 30 min prior to plating. Cultures were incubated for an additional 2, 3.5, 4.5 or 24 h before cells were fixed and stained for TRAP activity. The number of attached osteoclasts was determined by counting the number of TRAP-positive multinucleated cells using a light microscope at 200× magnification.

Experiments to investigate the effects of M-CSF and Ca^{2+} on Vitaxin-induced inhibition of osteoclast attachment were done by plating 250 μl aliquots of cell suspension in 12-well plastic culture dishes, and incubating for 1 h at 37°C and 5% CO_2. Subsequently, 1 ml of

supplemented α-MEM was added either with or without 2.5 mM Ca^{2+} or 50 ng/ml M-CSF, followed by an 18-h incubation. Cultures were then washed gently with α-MEM to remove non-attached cells and further incubated in the absence or presence of Vitaxin (300 ng/ml) for 48 h. Cells were stained for TRAP and counted.

Experiments aimed at examining the signaling pathway mediating the effects of M-CSF were done using the PI3-kinase inhibitor, wortmannin. Briefly, osteoclast cultures grown for 48 h were washed with Ca-free Hank's balanced salt solution before they were incubated in PBS +4% FBS with or without 500 nM wortmannin at 4°C for 1 h. This was followed by incubating the cultures in presence or absence of 50 ng/ml M-CSF and/or 300 ng/ml Vitaxin for an additional 1.5 h at 37°C and 5% CO_2 before the experiment was stopped and osteoclasts were fixed.

Time-Lapse Microscopy

Morphology and motility of osteoclasts were assessed using time-lapse video microscopy as previously described [Nakamura et al., 1999]. Briefly, osteoclasts were either untreated or treated for 30 min with 100 ng/ml of M-CSF. After the pretreatment period, Vitaxin (100–1,000 ng/ml) was added and the effects on osteoclasts were monitored for another 30 min. To quantify responses, the periphery of each cell was outlined and the planar cell area was calculated at 5 min intervals using digital image analysis. Data were normalized as a percentage of the initial area.

Resorption Studies on Bovine Bone Slices

Aliquots (100 μl) of the initially isolated cell suspension were plated on cortical bone slices in 96-well plates. After an 18-h attachment period, the cultures were washed gently with α-MEM to remove unattached cells and then incubated for an additional 48 h in the presence or absence of Vitaxin. Cells were then fixed and stained. Immunohistochemical staining for collagen type I was used to identify resorption lacunae as previously described [Shibutani and Heersche, 1993].

Resorption Studies Using the Osteologic Bone Cell Culture System

Resorption assays were also performed on 16-well osteologic slides using the same experimental design and culture conditions described for bone slices. Following the 48-h culture period, cells were detached from the surface of osteologic slides according to the manufacturer's recommendation. Briefly, 200 μl of bleach solution (6% NaOCl, 5.2% NaCl) was added to wells before removing media and the slides were agitated for 5 min. Wells were washed 2× with 150 μl dH_2O and then examined under the light microscope to ensure complete removal of cells. The contrast between non-resorbed areas and resorption pits was visualized using von Kossa staining. Briefly, 150 μl 3% $AgNO_3$ was added to the wells for 18 min in the dark. Wells were thoroughly washed 5× with 150 μl dH_2O, then developed in 0.5% hydroquinone for 3 min. Wells were washed again 5× with 150 μl dH_2O then fixed using 150 μl 2% $Na_2S_2O_3{\cdot}5H_2O$. The total area of resorption and number of resorbed regions were quantified using an ImagePro® analysis system.

Statistics

Statistics were carried out using SPSS 12.0 for Windows using one way analysis of variance (ANOVA) and Dunnette T3 test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Vitaxin Inhibits Osteoclast Resorption

We first evaluated the effect of Vitaxin on osteoclastic resorption using osteologic slides. Rabbit osteoclasts were left to attach for 18 h then incubated with Vitaxin at 30, 100, and 300 ng/ml for an additional 24 h. Resorption was significantly decreased by ~55% at 100 and 300 ng/ml, whereas 30 ng/ml Vitaxin resulted in a slight increase in resorptive activity (Fig. 1). Although the increase at 30 ng/ml was not statistically significant, this observation is consistent with the observation that αvβ3 antagonists at low concentrations can cause the activation of the integrin [Legler et al., 2001].

Vitaxin Decreases the Number of Osteoclasts Attached to Plastic

To address whether the decrease in resorption was due to a decrease in osteoclast number, the number of osteoclasts that remained attached after treatment with Vitaxin was examined. Osteoclasts were cultured on plastic

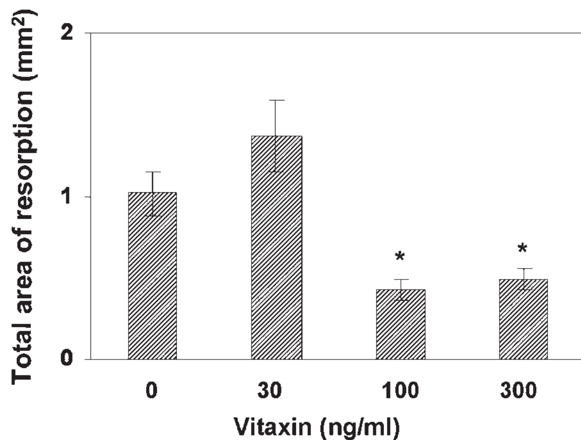

Fig. 1. Vitaxin decreases osteoclast resorption on osteologic slides. Rabbit osteoclasts were cultured on osteologic slides for 18 h before adding Vitaxin (30–300 ng/ml) for an additional 24 h. Non-resorbed areas were visualized using von Kossa staining. The surface area of pits was outlined manually and measured using ImagePro software system. Each data point represents the pooled results from four discs per treatment and is expressed as total area of resorption per well (means ± SEM). *P-value < 0.05 versus control group. Similar results were obtained in two separate experiments.

for 18 h before introducing Vitaxin (30, 100 and 300 ng/ml) for an additional 48 h. A decrease in the total number of osteoclasts attached was observed with a maximum of ~35% inhibition at 300 ng/ml (Fig. 2A). The addition of fresh Vitaxin in the middle of the 48-h incubation made no difference to the results (data not shown) suggesting that there was no proteolytic degradation of the antibody over the 48-h culture period.

Osteoclasts form and enlarge through the process of fusion. To determine whether the decrease in osteoclast number was due to increased fusion of existing multinucleated osteoclasts, the total number of nuclei in the experiment was quantified by hematoxylin staining. The decrease in the total number of nuclei paralleled that seen in the number of osteoclasts (Fig. 2B), suggesting that the decrease in osteoclast number is not a result of increased fusion.

Vitaxin Preferentially Inhibits the Attachment of Small Osteoclasts (<10 Nuclei)

Substantial differences exist between large and small osteoclasts, as defined by their number of nuclei, with respect to their resorptive activity and pH regulation [Lees et al., 2001]. We decided to determine whether Vitaxin differentially affected one of the two distinct cell populations. Figure 2C shows that Vitaxin decreased the number of small

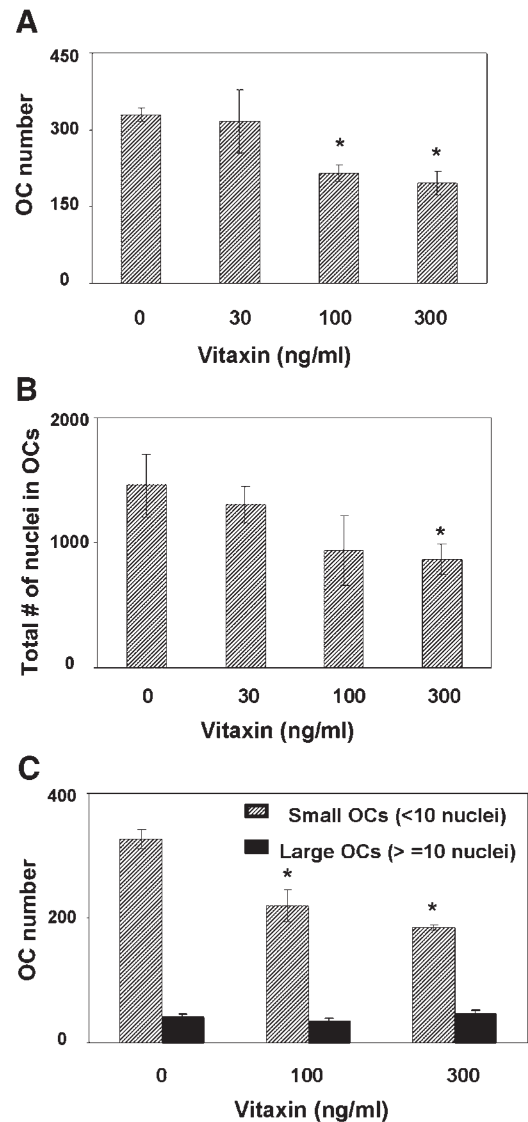

Fig. 2. Vitaxin decreases the attachment of small osteoclasts (OCs) (<10 nuclei) on plastic. Rabbit osteoclasts were cultured in 6-well plates for 18 h before adding Vitaxin (30–300 ng/ml) for an additional 48 h. Cells were (**A**) stained for TRAP activity or (**B**) nuclei were stained using hematoxylin. The number of TRAP + osteoclasts attached to plastic and the total number of nuclei in osteoclasts were counted using a light microscope at 100× magnification. **C**: The total number of TRAP + osteoclasts and the number of TRAP-positive osteoclasts with ≥10 nuclei were counted using a light microscope at 200× magnification. Each data point represents the pooled results from three wells per treatment and is expressed as total number per well. Data are means ± SEM. Similar results were obtained in three other experiments. *P-value <0.05 versus control group.

osteoclasts by an average of 35% whereas its effect on large osteoclasts was negligible. Vitaxin's preferential inhibition of small osteoclasts may reflect the differences in αvβ3 expression noted between large and small osteoclasts [Trebec et al., in press].

Vitaxin Does not Affect the Resorptive Activity of Attached Osteoclasts

The results described above show that the inhibition of osteoclastic resorption by Vitaxin is explained at least in part by the decrease in the number of attached osteoclasts. We next asked whether Vitaxin might also interfere with the resorptive capacity of individual osteoclasts that remained attached. Similar to our findings with hydroxyapatite-coated slides (Fig. 1), incubation of osteoclasts on bone slices with Vitaxin (100 ng/ml) for 48 h decreased both the total area of resorption as well as the number of osteoclasts attached by 50% (Fig. 3A,B). When the resorptive activity per osteoclast was calculated, we found that treatment with Vitaxin had no significant effect (Fig. 3C), suggesting that Vitaxin does not affect the resorptive capacity of attached osteoclasts but rather affects osteoclast attachment and/or formation.

Vitaxin Inhibits Attachment but Not Early Stages of Osteoclast Formation

Since αvβ3 plays a role in osteoclastogenesis [Teitelbaum and Ross, 2003], the effect of Vitaxin was determined on early stages of osteoclast formation and multinucleation. To distinguish between actions on osteoclast attachment and formation, we examined the effects of pre-incubation with Vitaxin on attachment of osteoclasts after 2 h. We also examined the effects of Vitaxin on osteoclast number following incubation in the presence or absence of Vitaxin up to 24 h to assess formation. When the freshly isolated cell suspension was incubated with Vitaxin (300 ng/ml) at 4°C for 30 min prior to plating, osteoclast attachment measured at 2 h was decreased by ~40% (Fig. 4A). When the cells were cultured for a 3.5, 4.5, and 24 h, we observed a gradual and similar increase in the number of multinucleated osteoclasts in both the control and the Vitaxin-treated groups (Fig. 4B). Micrographs from this experiment illustrate that neither the number of preosteoclasts nor the size of the aggregates they formed prior to fusing was altered by blocking αvβ3 (Fig. 4C). When the percentage increase in osteoclast number was calculated between the 2 and 24 h time points and combined from three different experiments, there was no significant difference between the control and the Vitaxin-treated groups (data not shown). These data are compatible with the view that the major effect of Vitaxin is on osteoclast attachment, rather than formation and multinucleation.

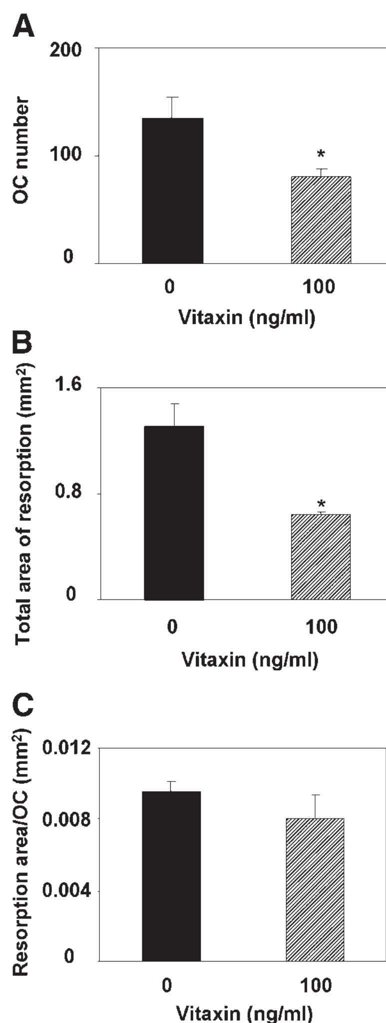

Fig. 3. Vitaxin decreases osteoclast (OC) attachment and resorption on bone but does not affect resorbed area per osteoclast. Rabbit osteoclasts were cultured on bone slices for 18 h before Vitaxin (100 ng/ml) was added for an additional 48 h. The number of TRAP-positive osteoclasts attached to bone slices was counted using a light microscope at 250× magnification (**A**). Resorption pits were visualized using collagen type I staining. The surface area of pits was outlined and measured using ImagePro software system (**B**). and the resorbed area per osteoclast was calculated (**C**). Each data point represents the pooled results from six bone slices per treatment and is expressed per well. Similar results were obtained in two separate experiments. Data are the means ± SEM. *P-value <0.05 versus control group.

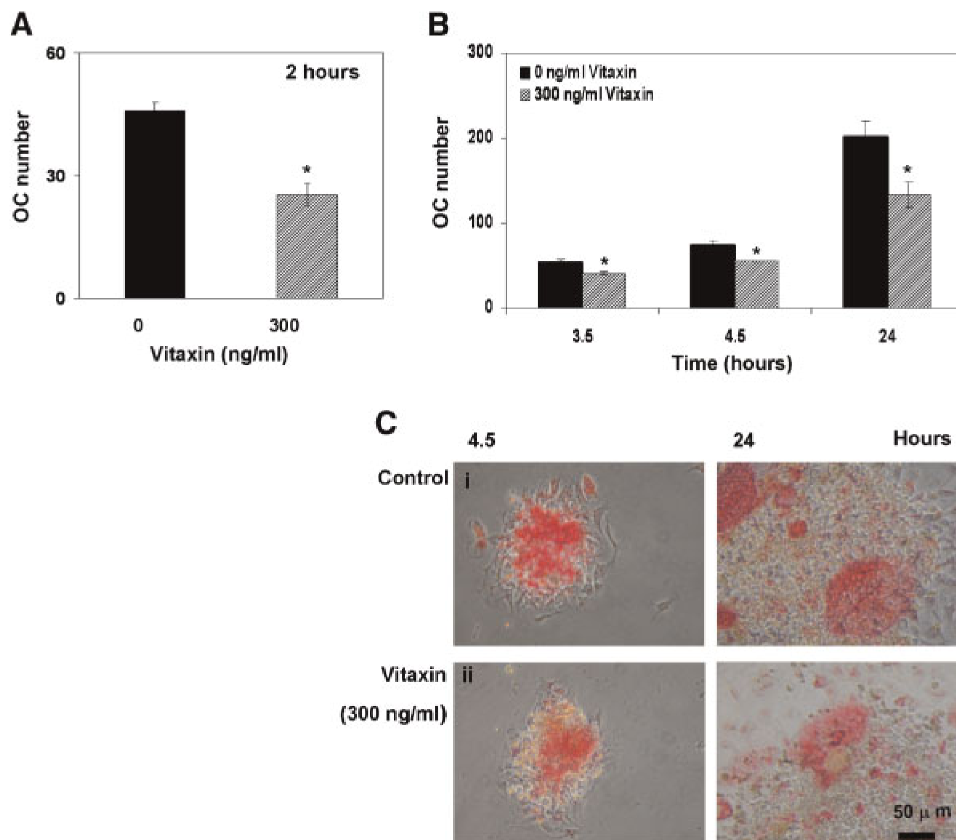

Fig. 4. Vitaxin decreases osteoclast (OC) initial attachment but does not affect osteoclast formation. Rabbit osteoclasts were resuspended in media with or without 300 ng/ml Vitaxin for 30 min at 4°C, then plated in 6-well plates and incubated for an additional 2 h (**A**), 3.5, 4.5, and 24 h (**B**) under standard culture conditions in the continued presence or absence of Vitaxin. Cells were stained for TRAP activity and the number of TRAP-positive osteoclasts was counted. Each data point represents the pooled results from three wells per treatment and is expressed per well. Data are means ± SEM. Similar results were obtained in two other experiments. **P*-value < 0.05 versus control group. **C**: Micrographs show that 300 ng/ml Vitaxin does not affect the number of preosteoclasts or the size of the aggregates they formed over a 24 h culture period in comparison to the control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Vitaxin Causes Retraction of Osteoclasts Only in the Presence of M-CSF

Cell morphology studies were done to assess Vitaxin's effect on osteoclast spreading. When osteoclasts were treated with Vitaxin (100 ng/ml) alone, no significant changes were observed in their planar area during a 25 min treatment period (Fig. 5B). In contrast, after treating cultures with 100 ng/ml M-CSF, Vitaxin significantly decreased the osteoclast planar area by 25% within 5 min (Fig. 5B,C). Figure 5A comprises micrographs taken during the experiment and is representative of the change that occurred in osteoclast morphology after treatment with M-CSF and Vitaxin.

Vitaxin's Effect on Attachment is Altered by Factors Known to Change the Conformation of $\alpha v\beta 3$

Integrins alternate between an inactivated and an activated conformation, with the activated form having a higher ligand affinity [Xiong et al., 2001, 2002]. Growth factors, divalent cations, and anti-ligand induced binding site antibodies (anti-LIBS) [Frelinger et al., 1991] all shift the integrin from one form to the other and therefore affect cell attachment and spreading. It has been shown that M-CSF increases, whereas high concentrations of Ca^{2+} decreases the number of activated $\alpha v\beta 3$ receptors on the plasma membrane of osteoclasts [Faccio et al., 1998, 2003]. To assess

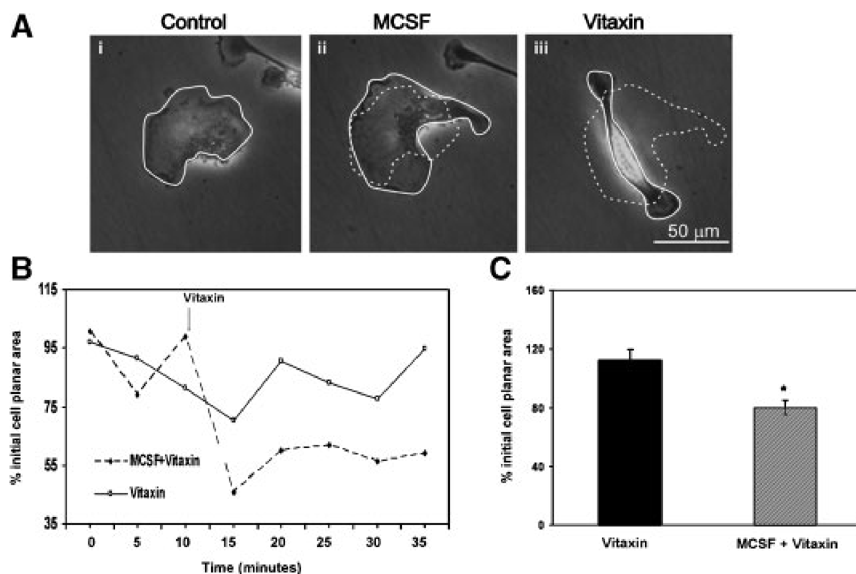

Fig. 5. Vitaxin decreases osteoclast (OC) planar area only when cultures are pretreated with M-CSF. Isolated rabbit osteoclasts were cultured for 24 h before they were incubated for 30 min in the presence or absence of 100 ng/ml of M-CSF. After the pretreatment period, Vitaxin (100 ng/ml) was added and planar cell area was monitored for another 25 min. **A**: The series of video micrographs shows the response of an M-CSF-treated osteoclast to Vitaxin. Frame i shows an osteoclast before addition of M-CSF (100 ng/ml). Frame ii shows the same osteoclast after addition of M-CSF. Dotted line represents the outline of the cell in Frame i superimposed on Frame ii. Frame iii shows retraction after addition of Vitaxin (100 ng/ml). Dotted line represents the outline of the cell in Frame ii superimposed on Frame iii. To quantify retraction induced by the antibody, the periphery of each cell was outlined and the planar cell area was calculated at 5 min intervals using digital image analysis and normalized to the initial area (**B**). **Panel C** represents the planar area of osteoclasts, 5 min after adding Vitaxin to the cultures. Data are means ± SEM of 12 osteoclasts from three independent experiments. $^{*}P < 0.05$.

if Vitaxin is dependent on the integrin's conformational state, osteoclasts were incubated with either M-CSF (50 ng/ml) or Ca^{2+} (2.5 mM Ca^{2+} total) for 18 h before the addition of 300 ng/ml Vitaxin for a further 48 h of culture. Vitaxin in the presence of M-CSF resulted in a $55 \pm 4\%$ decrease in osteoclast number compared to only $33 \pm 3\%$ decrease in the Vitaxin only treated group (% change was calculated based on results obtained from three independent experiments). In contrast, in the presence of 2.5 mM Ca^{2+}, there was no inhibition in the Vitaxin-treated group (Fig. 6A). These results suggest that Vitaxin preferentially inhibits the activated form of $\alpha v\beta 3$. To examine whether M-CSF's effects were mediated through PI3-kinase, we added a PI3-kinase inhibitor, wortmannin, for 1 h prior to stimulating the cultures with M-CSF. In Figure 6B, we again show that Vitaxin's inhibitory effect was increased in the presence of M-CSF, but the M-CSF-dependent increase was abolished by wortmannin. The presence of wortmannin alone did not significantly change Vitaxin's inhibitory effect (Fig. 6B).

DISCUSSION

Vitaxin, an anti-human $\alpha v\beta 3$ antibody is currently in clinical trials as a treatment for metastatic melanoma and prostate cancer [Mikecz, 2000; Tucker, 2006]. To help understand how Vitaxin prevents metastatic bone loss in cancer patients, the aim of this study was to elucidate the mechanism by which this therapeutic decreases bone resorption. Insights into how osteoclasts interact with the $\alpha v\beta 3$ antagonist are important for the process of developing new generations of treatment which are more effective and also to determine methods of using the antibody as a cancer imaging and drug delivery agent. Previous studies have shown that RGD mimetics that block the ligand-binding site of integrin $\alpha v\beta 3$ also inhibit bone resorption in vitro and in vivo [Chambers et al., 1986; Horton et al., 1991; Nakamura et al., 1996; Nakamura et al., 1999; Carron et al., 2000; Meissner et al., 2002]. The results reported here show that Vitaxin also inhibits bone resorption.

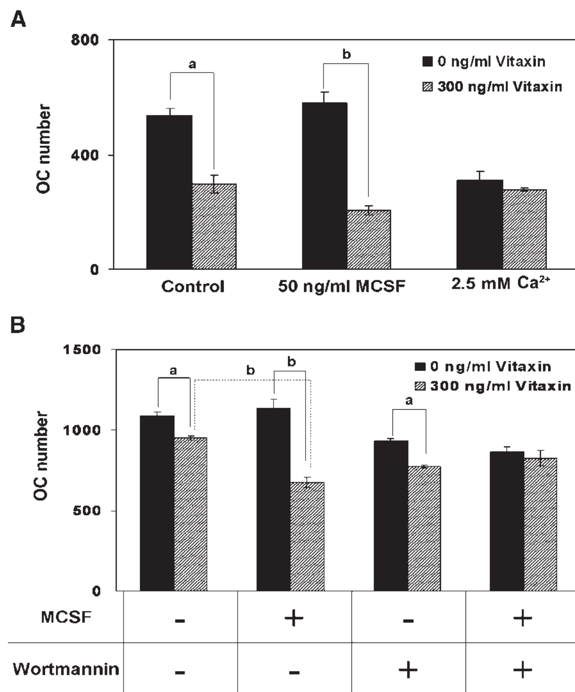

Fig. 6. Vitaxin's effect on attachment can be altered by factors affecting the conformation of αvβ3. **A**: Rabbit osteoclasts (OCs) were cultured in 12-well plates in the presence and absence of 50 ng/ml M-CSF (activates αvβ3) or 2.5 mM Ca^{2+} (inactivates αvβ3) for 18 h before adding 300 ng/ml Vitaxin for an additional 48 h. **B**: Osteoclasts were incubated in the presence or absence of 500 nM wortmannin (PI3-kinase inhibitor which blocks M-CSF induced activation) for 1 h at 4°C. Cultures were subsequently incubated in the presence or absence of 50 ng/ml M-CSF with or without 300 ng/ml Vitaxin for 1.5 h at 37°C 5% CO_2. Cells were stained for TRAP and the number of TRAP-positive osteoclasts was counted. Each data point represents the pooled results from four wells per treatment and is expressed as total number of osteoclasts per well. Data are means ± SEM. Similar results were obtained in two other experiments. a: *P*-value <0.05 and b: *P*-value <0.01.

To study whether the inhibition of resorption was caused by a reduction in the number of resorbing osteoclasts, we examined the effect of Vitaxin on the number of attached osteoclasts. We found a decrease in the number of osteoclasts which paralleled the decrease in resorption.

Since the decrease in the number of osteoclasts could result from fusion of smaller osteoclasts into larger ones, the total number of nuclei in TRAP-positive cells was determined. The decrease in the number of nuclei corresponded to the decrease in the number of osteoclasts, indicating that the reduction in osteoclast number was not due to an effect on fusion. This is consistent with the results of Nakamura et al., 1999 who showed that echistatin inhibited resorption through impairment of osteoclast attachment accompanied by a decrease in both actin ring formation and osteoclast spreading. However, in another series of experiments, echistatin in vivo was found to increase the number of morphologically normal osteoclasts but still prevented bone loss in mice with secondary hyperparathyroidism [Masarachia et al., 1998]. The authors suggested that the reduction in bone turnover resulted from dysfunctional and inefficient osteoclasts, which does not seem to be the case in our experiments. In our resorption studies, Vitaxin was shown not to diminish the ability of osteoclasts to degrade bone.

Our results also indicate that Vitaxin primarily affects small osteoclasts containing <10 nuclei and has no effect on large osteoclasts containing ≥10 nuclei. Comparison between osteoclast populations based on their size evolved from the observation that large osteoclasts were prevalent in RA and Paget's disease. This observation led to the examination of the characteristics of those two groups of osteoclasts. Previous data had shown that large osteoclasts are more actively resorbing than small osteoclasts and both groups use different methods to regulate their cytoplasmic pH [Lees and Heersche, 2000; Lees et al., 2001]. Large osteoclasts were also found to express an average of threefold higher levels of αvβ3 than small osteoclasts [Trebec et al., in press]. Vitaxin's preferential effect on small osteoclasts could be caused by the increased levels of αvβ3 on the surface of large osteoclasts, making them less susceptible to detachment by Vitaxin. Alternatively, large osteoclasts might be using an αvβ3-independent mechanism for attachment. These data also show that Vitaxin does not interfere with osteoclast multinucleation. In contrast, echistatin was found to inhibit both multinucleation and migration in an osteoblast/osteoclast coculture system [Nakamura et al., 1998]. These different results can be attributed to the different models and compounds used as well as how both osteoclast formation and multinucleation were measured.

Monitoring osteoclast formation from 2 to 24 h after plating revealed that Vitaxin inhibited initial attachment but did not affect the rate of osteoclast formation. The role of the integrin αvβ3 in osteoclast formation has long been debated. In vitro, bone marrow macrophages (BMMs) derived from mice with the Glanzmann

mutation in the β3 subunit, formed fewer osteoclasts with abnormal structure and function [Feng et al., 2001]. Similar results were reported for osteoclasts differentiated from β3 null BMMs [Faccio et al., 2003]. In vivo, β3 null mice, as well as those treated with echistatin in a hyperparathyroidism model, all showed increases in the numbers of osteoclasts while resorption was inhibited. This paradox was solved when it was noticed that levels of M-CSF in the sera of those animals was elevated. This was followed by a series of experiments to elucidate the effect of M-CSF on osteoclasts in vitro. High concentrations of M-CSF added to β3−/− BMM cultures were shown to rescue the formation of osteoclasts. Both the activation of extracellular signal-regulated kinase as well as the expression of the transcription factor c-Fos, which are essential for osteoclastogenesis, were found to be at lower than normal levels and those levels were restored by M-CSF along with osteoclast formation [Masarachia et al., 1998; McHugh et al., 2000]. These results infer an overlap between the signaling pathways of c-Fms and αvβ3 during osteoclast formation explaining how M-CSF compensates for the absence of β3. A similar compensatory mechanism might be occurring in our mixed culture system, and may explain why Vitaxin did not affect osteoclast formation rates.

Conformational changes occurring in the cytoplasmic domain of integrins are a source of their versatility [Xiong et al., 2001, 2002; Mould et al., 2002]. When integrins change their form from bent to flexed, they are said to be in an activated state. This activation state is responsible for modulation of their affinity for ligands and is accompanied by a more stable interaction with the RGD sequence of matrix proteins, which in turn affects attachment and cell spreading [Pelletier et al., 1995; Kim et al., 2003]. Since αvβ3 activation is needed for osteoclast activation, polarization, and resorption, we wanted to find out whether modulating αvβ3's structure could alter Vitaxin's effect on osteoclast adhesion. Inactivation and activation of αvβ3 occur through different mechanisms including binding of divalent cations such as Ca^{2+}, Mn^{2+}, and Mg^{2+} [Spillmann et al., 2002], activating antibodies [Faccio et al., 1998], low antagonist concentrations [Legler et al., 2001], and growth factors [Faccio et al., 2002]. M-CSF, known to activate αvβ3, enhanced Vitaxin's inhibitory effect; whereas elevation of extracellular Ca^{2+}, known to shift integrins to their inactive state, abolished those effects. The PI3-kinase inhibitor wortmannin abolished M-CSF's effects on the action of Vitaxin, suggesting that they are mediated though the PI3-kinase signaling pathway. These findings suggest that Vitaxin might differentially recognize and bind the activated form of αvβ3. In support of Vitaxin preferentially binding to activated αvβ3 is the observation that a reduction of osteoclast planar area with Vitaxin only occurred when the osteoclasts were pretreated with M-CSF. These data are in agreement with studies showing that LM609 reduced osteoclast spreading only in the presence of M-CSF [Teti et al., 1998].

Our study is the first to show that Vitaxin decreases bone resorption but does not affect resorption area per osteoclast or their rate of formation. Rather, the decrease in bone resorption was shown to result from an initial reduction in osteoclast attachment. Furthermore, our data suggests that Vitaxin's inhibitory effects are mediated by the interaction with the activated form of αvβ3 and that the PI3-kinase signaling pathway is involved in this process. These findings are important as they suggest strategies to enhance the therapeutic potentials of Vitaxin as well as other integrin antagonists currently in clinical use.

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