The Adhesive and Migratory Effects of Osteopontin Are Mediated via Distinct Cell Surface Integrins

Role of $\alpha_v\beta_3$ in Smooth Muscle Cell Migration to Osteopontin In Vitro

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Abstract

Osteopontin is an arginine-glycine-aspartate containing acidic glycoprotein postulated to mediate adhesion, migration, and biomineralization in diverse tissues. The mechanisms explaining this multifunctionality are not well understood, although it is known that one osteopontin receptor is the $\alpha_v\beta_3$ integrin. In this work, we studied human smooth muscle cells varying in $\alpha_v\beta_3$ levels to identify additional osteopontin receptors. We report that, in addition to $\alpha_v\beta_3$, both $\alpha_v\beta_5$ and $\alpha_v\beta_1$ are osteopontin receptors. Moreover, the presence or absence of $\alpha_v\beta_3$ on the cell surface altered the adhesive and migratory responses of smooth muscle cells to osteopontin. Adhesion of $\alpha_v\beta_3$-deficient cell populations to osteopontin was only half that of cells containing $\alpha_v\beta_3$, and migration toward an osteopontin gradient in the Boyden chamber was dependent on cell surface $\alpha_v\beta_3$. Although $\alpha_v\beta_3$-deficient smooth muscle cells were unable to migrate to osteopontin, they did migrate significantly in response to vitronectin and fibronectin. These findings represent the first description of $\alpha_v\beta_5$ and $\alpha_v\beta_1$ as osteopontin receptors and suggest that, while adhesion to osteopontin is supported by integrins containing β_1, β_3, and β_5, migration in response to osteopontin appears to depend on $\alpha_v\beta_3$. Thus, interaction with distinct receptors is one mechanism by which osteopontin may initiate multiple functions. (*J. Clin. Invest.* 1995. 95:713–724.) Key words: osteopontin • integrin • migration • smooth muscle cell • adhesion

Introduction

Smooth muscle cell adhesion and migration are critical for development of visceral and vascular muscles, as well as the fibromuscular lesions commonly associated with atherosclerosis, hypertension, and vascular wound repair. A role for osteopontin in these processes was suggested recently by its marked upregulation in healing arterial wounds and its abundance in human atherosclerotic plaques (1–3). Furthermore, osteopontin is an adhesive stimulus for both vascular (4) and intestinal (5) smooth muscle and promotes migration of smooth muscle cells in a gradient-dependent fashion (4). These findings prompted our investigation of the mechanisms by which osteopontin supports these two functions in vascular smooth muscle.

Osteopontin is an highly acidic, secreted glycoprotein containing the adhesive motif, arginine-glycine-aspartate (RGD)[1] (for review see reference 6). Hexamer peptides containing the RGD sequence were shown to block bone, epithelial, and smooth muscle cell adhesion to osteopontin (5, 7, 8), and later the integrin $\alpha_v\beta_3$ was identified as an osteopontin receptor in osteoclasts (9–11). Likewise, we recently found that human smooth muscle cells could use $\alpha_v\beta_3$ as an adhesive receptor (4). However, a neutralizing anti-$\alpha_v\beta_3$ antibody was only able to partially block cell adhesion to osteopontin, whereas an RGD-containing hexamer peptide could completely inhibit this interaction. This suggested that other RGD-dependent receptors, most likely integrins, were also involved as cell surface osteopontin receptors.

In this report, human aortic smooth muscle cells with variable expression of cell surface $\alpha_v\beta_3$ integrin were used to test the hypothesis that osteopontin can interact with cell surface receptors other than $\alpha_v\beta_3$. We demonstrate that cultured human smooth muscle cells deficient in $\alpha_v\beta_3$ integrin will adhere and spread on substrates of osteopontin, and that these interactions are mediated by the integrins $\alpha_v\beta_5$ and $\alpha_v\beta_1$. These cells, however, are incapable of migrating toward osteopontin. In contrast, smooth muscle cells containing significant levels of $\alpha_v\beta_3$ are capable both of adhering and migrating to osteopontin. These results demonstrate that osteopontin can interact with three different receptors which in turn mediate different cellular responses to osteopontin.

Methods

Cell culture. Adult human aortic medial smooth muscle cells were obtained from heart transplant donor specimens using a collagenase/elastase digestion method (12) and cultured in Waymouth's MB 752/1 medium (GIBCO BRL, Grand Island, NY) containing 0.23% sodium bicarbonate, 0.35 mg/ml L-glutamine, 1 mM sodium pyruvate, and 0.01 mM nonessential amino acids supplemented with 20% fetal bovine serum (Hyclone Labs, Logan, UT) and 100 U/ml each penicillin and streptomycin (GIBCO BRL). Cells derived in this fashion were judged to be smooth muscle by immunofluorescence staining with an anti-

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1. *Abbreviations used in this paper:* HPF, high power fields; Pen, penicillamine; RGD, arginine-glycine-aspartate.

smooth muscle alpha actin specific antibody, SM-1 (Sigma Immunochemicals, St. Louis, MO). An adult human smooth muscle cell line was derived as above and retrovirally transfected with human papilloma virus proteins E5 and E6 and was characterized previously (13). Human newborn aortic smooth muscle cells were derived from a newborn infant autopsy (2-d-old infant) specimen obtained from Dr. Joe Siebert (Children's Hospital, Seattle, WA) and characterized as above. Cells were fed twice weekly and passaged by trypsinization at confluence. Cultures were maintained in a humidified incubator with 5% CO_2/95% air at 37°C. Adult smooth muscle cells were used between passages 5 and 7, newborn smooth muscle cells were used between passages 6 and 8, and retrovirally transfected smooth muscle cells were used between passages 16 and 23. We have used three independent isolates of human smooth muscle cells with low β_3 integrin and similar integrin profiles in these experiments with consistent results. The smooth muscle cell strain derived from newborn (2-d) aorta was the only isolate identified in this study with significant levels of $\alpha_v\beta_3$ integrin. Recently however, using RNA analysis, we have in addition consistently found that β_3 is present in fetal human smooth muscle cell samples (our unpublished observations).

Adhesive proteins. Osteopontin was purified from conditioned medium of rat pup smooth muscle cell cultures as described previously (4). Human vitronectin (Sigma Immunochemicals) was resuspended in phosphate-buffered saline (PBS) to a concentration of 0.5 mg/ml and stored frozen until use. Human fibronectin was purchased from GIBCO BRL and stored frozen at a concentration of 0.5 mg/ml in PBS until use. Vitrogen 100 (Celtrix Labs, Palo Alto, CA) is purified collagen from bovine dermis containing > 95% type I collagen and was used for adhesion studies on collagen.

Antibodies and peptides. Antibodies against human integrins $\alpha_v\beta_5$ (clones P1F6 and P3G2) (14), β_1 (clone P4C10) (15), $\alpha_v\beta_3$ (clone LM609) (16), α_2 (clone P1E6) (17), α_5 (clone P1D6) (18), α_3 (clone P1B5) (19), α_v (20), and α_2 (clone P1H5) (18) were purchased from GIBCO BRL. Human antimelanoma antibody L230 was received in supernatant form from Dr. Robert Pytela (UCSF, San Francisco, CA) and has been characterized to be directed against the α_v subunit (21). Antibodies against α_v (clone AMF/7) and β_3 (clone SZ 21) were purchased from Amac, Inc. (Westbrook, ME), and anti-β_3 (clone Y2/51) was purchased from Dako Corp. (Carpinteria, CA). Anti-α_v (MAB 1980) was purchased from Chemicon International, Inc. (Temecula, CA), and antifibronectin antibody (clone II) was purchased from GIBCO BRL. Antibodies above were tested at several dilutions of ascites in inhibitory assays, and the concentration used in these studies (1:500 dilution of ascites, unless otherwise stated) gave the greatest inhibitory activity in our assays. Purified LM609 IgG was used in some experiments at concentrations noted. Monoclonal antivinculin clone VIN-11-5 was purchased from Sigma Immunochemicals, and fluorescein-conjugated rabbit immunoglobulin to mouse immunoglobulins was purchased from Dako Corp. OP199 is a goat polyclonal antibody made against rat smooth muscle cell–derived osteopontin that has been characterized previously (4). Synthetic peptides used were purchased from GIBCO BRL and corresponded to sequences GRGDSP, GRGESP, and GPenGRGDSPCA (PenRGD), where Pen stands for penicillamine. The PenRGD peptide specifically inhibits vitronectin receptors and does not interact with fibronectin receptors (22, 23). The peptides were dissolved in PBS and stored frozen until use.

Adhesion assay. Adhesion studies were performed as described previously (4). Briefly, test adhesive substrates were diluted in PBS to stated concentrations and 50 μl/well was added to 96-well Maxisorp plates (Nunc, Inc., Naperville, IL) and placed overnight at 4°C. Under these conditions, coating efficiencies for osteopontin, vitronectin, and fibronectin were previously determined to be 38, 27, and 37%, respectively (4). Wells were rinsed with PBS, and nonspecific binding sites were blocked by the addition of 10 mg/ml bovine serum albumin (BSA) in PBS for 1 h at 37°C. Control wells contained BSA alone. Cells grown to confluence under the conditions described above were minimally trypsinized (1–2 min), and were added to an equal volume of 0.5 mg/ml serum trypsin inhibitor (Sigma Immunochemicals). Cells were centrifuged and resuspended in Waymouth's serum–free medium with 1 mg/ml BSA and plated into wells at 30,000 cells/well. In experiments where neutralizing antibodies were used, they were diluted into the cell suspension immediately before plating of cells into wells. Plates were incubated for 60 min at 37°C, and the assay was terminated by rinsing the plates with PBS and fixing the cells with 4% paraformaldehyde. Cells were stained with 0.5% toluidine blue in 4% paraformaldehyde for 5 min and rinsed in water. They were then solubilized by the addition of 1% SDS and quantitated in a microtiter plate reader at 595 nm. We have previously demonstrated that this procedure of quantitation gives values proportional to the number of adherent cells (4). We have also demonstrated that the adhesive interaction of smooth muscle cells with osteopontin can occur without protein synthesis, and that both adhesion and focal contact formation occur in the presence of cycloheximide (4). All experiments were repeated independently at least three times, and groups of quadruplicates were analyzed. Data are expressed as means±SEM.

Statistics. Since several groups of antibody-treated cells were analyzed for significant differences in adhesion, Scheffé's F test for multiple comparisons was used.

Migration assay. Migration assays were performed essentially as described previously (4). Briefly, a polycarbonate filter with 8-μm pores (Costar Corp., Cambridge, MA) was coated on both sides overnight with 100 μg/ml human fibronectin at 4°C. Test substances were diluted to appropriate concentrations in Waymouth's complete medium containing 200 μg/ml BSA and were placed in the bottom wells of a modified Boyden chemotaxis chamber (NeuroProbe, Cabin John, MD). The polycarbonate filter was air dried and placed between the test proteins and the upper chamber. Human smooth muscle cells were grown to confluence under the conditions described above, were trypsinized briefly (1–2 min), and were added to an equal volume of 0.5 mg/ml soybean trypsin inhibitor. Cells were centrifuged and resuspended in equal volumes of 0.5 mg/ml soybean trypsin inhibitor and resuspended in Waymouth's serum-free medium containing 200 μg/ml BSA. Cells were added to upper chambers at 20,000 cells/well. For antibody inhibition studies, appropriate concentrations were established both in the upper and lower chambers. Migration was allowed to proceed for 5 h at 37°C in a humidified cell culture incubator, unless otherwise stated. After the incubation period, cells that had migrated to the bottom of the filter were fixed with methanol and stained with hematoxylin. Migration was quantitated by cell counts of three random ×400 high power fields (HPF) in each well or as described. Each test group was performed in quadruplicate wells.

Analysis of cell surface integrins. Integrin profiles on smooth muscle cell surfaces were analyzed using flow cytometry. Confluent cultures of cells were trypsinized, washed in PBS containing 0.2% BSA and 0.1% azide, and resuspended at 10^6 cells/ml. Cells were fixed in 2% neutral buffered formalin for 20 min and washed again. Primary antibodies against integrins were incubated with ~ 10^5 cells/antibody at 4°C overnight. In control reactions, no primary antibody or an irrelevant isotype-matched antibody was added. Cells were then washed and incubated with phycoprobe PE anti–mouse IgG (BioMeda Corp., Foster City, CA) for 30 min at 4°C, washed, and analyzed for fluorescence using a flow cytometer (Coulter Epics Elite, Hialeah, FL). X-axes of data represent log fluorescent intensity, and y-axes are the cell number. Surface antigen expression detected by flow cytometric analysis was verified by radioimmunoprecipitation of ^{125}I surface-labeled proteins (24).

Immunocytochemistry. For vinculin staining, cells were fixed in 4% paraformaldehyde at room temperature for 15 min and permeabilized for 2 min with 0.2% Triton X-100 in 50 mM Tris, pH 7.5. Monoclonal antivinculin clone VIN-11-5 (Sigma Immunochemicals) was used at a 1:50 dilution and incubated for 1 h, followed by fluorescein-conjugated rabbit immunoglobulin to mouse immunoglobulins (Dako Corp.) at a 1:30 dilution for 1 h. Staining for integrins was done as above for vinculin using a 1:5 dilution of the monoclonal antibodies indicated, followed by a 1:30 dilution of fluorescein-conjugated rabbit immuno-

globulin to mouse immunoglobulins. All slides were mounted with Vectashield mounting media (Vector Labs, Inc., Burlingame, CA). Fluorescent samples were viewed under a Zeiss epifluorescent microscope.

Immunoprecipitation. Smooth muscle cells were trypsinized, counted, and resuspended in buffer containing 25 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, and 1.2 mM $CaCl_2$ (Hepes buffer). Cells were then lysed in a final concentration of 0.2 mM PMSF and 1% Triton X-100 for 30 min on ice with vortexing. The insoluble cell fragments were pelleted by centrifugation at 14,000 rpm for 10 min at 4°C, and the supernatant was precleared with protein A–Sephaose CL-4B (Pharmacia AB, Uppsala, Sweden) preswollen as per manufacturer's instructions. Proteins that bound nonspecifically were pelleted with the beads, and primary antibody P4C10 (2 μl of ascites) was added to the supernatant for 3 h at 4°C. Excess rabbit anti–mouse IgG was added for 30 min ($\sim$ 50 μg), followed by protein A–Sepharose CL-4B beads for an additional 30 min. Bound complexes were pelleted by centrifugation and solubilized by boiling in 50 mM Tris, pH 6.8, 5% SDS, 5% glycerol, 0.05% bromophenol blue, and 5% β-mercaptoethanol. Samples were electrophoresed on a 5% sodium dodecyl sulfate-polyacrylamide gel and transferred for immunoblotting.

Western blot analysis. Electrophoresed samples were transferred to a Polyscreen PVDF membrane (Dupont/NEN, Boston, MA) overnight and incubated at room temperature as follows. Nonspecific binding sites were blocked by 5% nonfat dry milk in a solution of 10 mM Tris, base pH 8, 150 mM NaCl, and 0.05% Tween 20 (TBS-T) for 1 h. All antibodies were diluted in TBS-T with 5% nonfat dry milk. Primary antibody anti-α_v (1:500 dilution) was added for 1 h, followed by extensive washings in TBS-T. Horseradish peroxidase–conjugated anti–mouse IgG (Promega Corp., Madison, WI) was added (1:2,000 dilution) for an additional hour followed by washes in TBS-T. Protein was visualized using the Renaissance Western blot chemiluminescence reagent (Dupont/NEN) as per manufacturer's instructions. Blot was exposed to Reflection autoradiography film (DuPont/NEN).

Results

Variable expression of $\alpha_v\beta_3$ by cultured human smooth muscle cells. While most cultured human smooth muscle cells express little or no $\alpha_v\beta_3$ integrin (Fig. 1 and reference 24), one aortic isolate derived from a 2-d-old infant expressed this antigen. In previous studies (4), we found that this particular isolate of smooth muscle cells ($\alpha_v\beta_3^+$) adhered to osteopontin via $\alpha_v\beta_3$, although this integrin could not account for all of the interaction of the cells with the substrate. To further characterize osteopontin/receptor interactions, integrin expression was analyzed on these cells and a variety of other smooth muscle cell strains. Using this approach, particular isolates were identified that differed in their surface levels of $\alpha_v\beta_3$ but had comparable expression of other integrin subunits.

Flow cytometric analysis of cell surface integrin expression in two different smooth muscle cell isolates is shown in Fig. 1. One strain (*left*) had very low levels of β_3 and $\alpha_v\beta_3$, consistent with the majority of smooth muscle cell cultures previously examined (24). The other isolate (*right*) differed, having significant levels of both β_3 and $\alpha_v\beta_3$ on the cell surface. The integrins β_1, α_v, and $\alpha_v\beta_5$ were comparable between the two isolates. These findings were consistent using a variety of anti-β_3 and anti-α_v antibodies. Both strains contained α_2, α_3, and α_5 integrin but neither cell strain contained detectable levels of α_4 or α_6 integrin (24). Results from radioimmunoprecipitation of cell surface proteins from these cell strains were in agreement with the flow cytometric analysis (24). These two smooth muscle cell isolates with either high $\alpha_v\beta_3$ levels ($\alpha_v\beta_3^+$) or deficient in $\alpha_v\beta_3$ ($\alpha_v\beta_3^-$) were chosen for further experiments to functionally address the importance of this particular integrin in cell interaction with osteopontin, as well as to identify additional osteopontin receptors.

Adhesion of $\alpha_v\beta_3^-$ and $\alpha_v\beta_3^+$ cells to osteopontin. Smooth muscle cells deficient in $\alpha_v\beta_3$ adhered to osteopontin substrates in a dose-dependent manner (Fig. 2 *A*) showing maximum adherence when a 3.25 μg/ml solution of osteopontin was used to coat wells. Of interest, maximum adhesion of $\alpha_v\beta_3^-$ cells on osteopontin was significantly less than cells bearing $\alpha_v\beta_3$ (Fig. 2 *A*) while adhesion of both cell types to the same molar concentration of fibronectin was equivalent (data not shown). In agreement with previous results, adhesion of $\alpha_v\beta_3^+$ cells to osteopontin was inhibited $\sim$ 50% with an antibody against $\alpha_v\beta_3$ (LM609) (4). In contrast, adhesion of $\alpha_v\beta_3^-$ cells was minimally affected by this antibody (Fig. 2 *B*). Therefore, receptors other than $\alpha_v\beta_3$ must be capable of promoting smooth muscle cell adhesion to osteopontin. Of particular interest, inclusion of anti-$\alpha_v\beta_3$ antibody in assays with $\alpha_v\beta_3^+$ cells reduced adhesion to a level similar to that of the cells deficient in this integrin (Fig. 2 *B*).

Although $\alpha_v\beta_3^-$ cells displayed a lower extent of attachment to osteopontin, adhesion was accompanied by cell spreading and cytoskeletal organization after 60 min (Fig. 3). These cells were well spread on the osteopontin substrate (Fig. 3 *A*). Immunofluorescence studies revealed that $\alpha_v\beta_3^-$ cells readily spread and formed focal contacts containing vinculin (Fig. 3 *B*). The morphology of cells as well as focal contact formation of $\alpha_v\beta_3^-$ cells were essentially identical to $\alpha_v\beta_3^+$ cells (see Fig. 9, *B* and *C*). These results indicated that surface proteins other than $\alpha_v\beta_3$ mediate binding to osteopontin, leading to cell spreading and cellular organization.

Osteopontin receptors in $\alpha_v\beta_3^-$ cells display properties characteristic of integrins. We used the hexamer peptides GRGDSP and GRGESP to determine if adhesion of $\alpha_v\beta_3^-$ cells to osteopontin was RGD dependent. The hexamer peptide containing the RGD sequence was able to inhibit cell adhesion to osteopontin in a dose-dependent manner, whereas the RGE-containing peptide had negligible effects (Fig. 5 *A*, *inset*). Furthermore, adhesion was abolished in the presence of EDTA, and was restored with increasing concentrations of either calcium or magnesium ions (data not shown). This requirement for divalent cations and dependence on the RGD sequence are consistent with the idea that osteopontin receptors may be other members of the integrin family distinct from $\alpha_v\beta_3$.

Multiple integrins are involved in smooth muscle cell adhesion to osteopontin. To determine which receptors participated in adhesion of $\alpha_v\beta_3^-$ and $\alpha_v\beta_3^+$ smooth muscle cells to osteopontin, we used a panel of neutralizing antibodies against integrins. Of the antibodies tested, those directed against $\alpha_v\beta_5$ as well as an antibody neutralizing all β_1 integrins consistently inhibited $\alpha_v\beta_3^-$ cell adhesion (Fig. 4 *A*). The inclusion of both anti-$\alpha_v\beta_5$ and anti-β_1 antibodies completely inhibited $\alpha_v\beta_3^-$ cell adhesion to osteopontin. The same concentrations of these antibodies alone inhibited $\alpha_v\beta_3^+$ cell adhesion to osteopontin $\sim$ 15–20%, but this decrease was not statistically different from control. The combination of the two antibodies did, however, significantly inhibit $\sim$ 40% of adhesion (Fig. 4 *B*). In addition, the inclusion of antibodies against the three integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and β_1 eliminated $\alpha_v\beta_3^+$ cell adhesion to osteopontin (Fig. 4 *B*), indicating that these three integrins account for the major

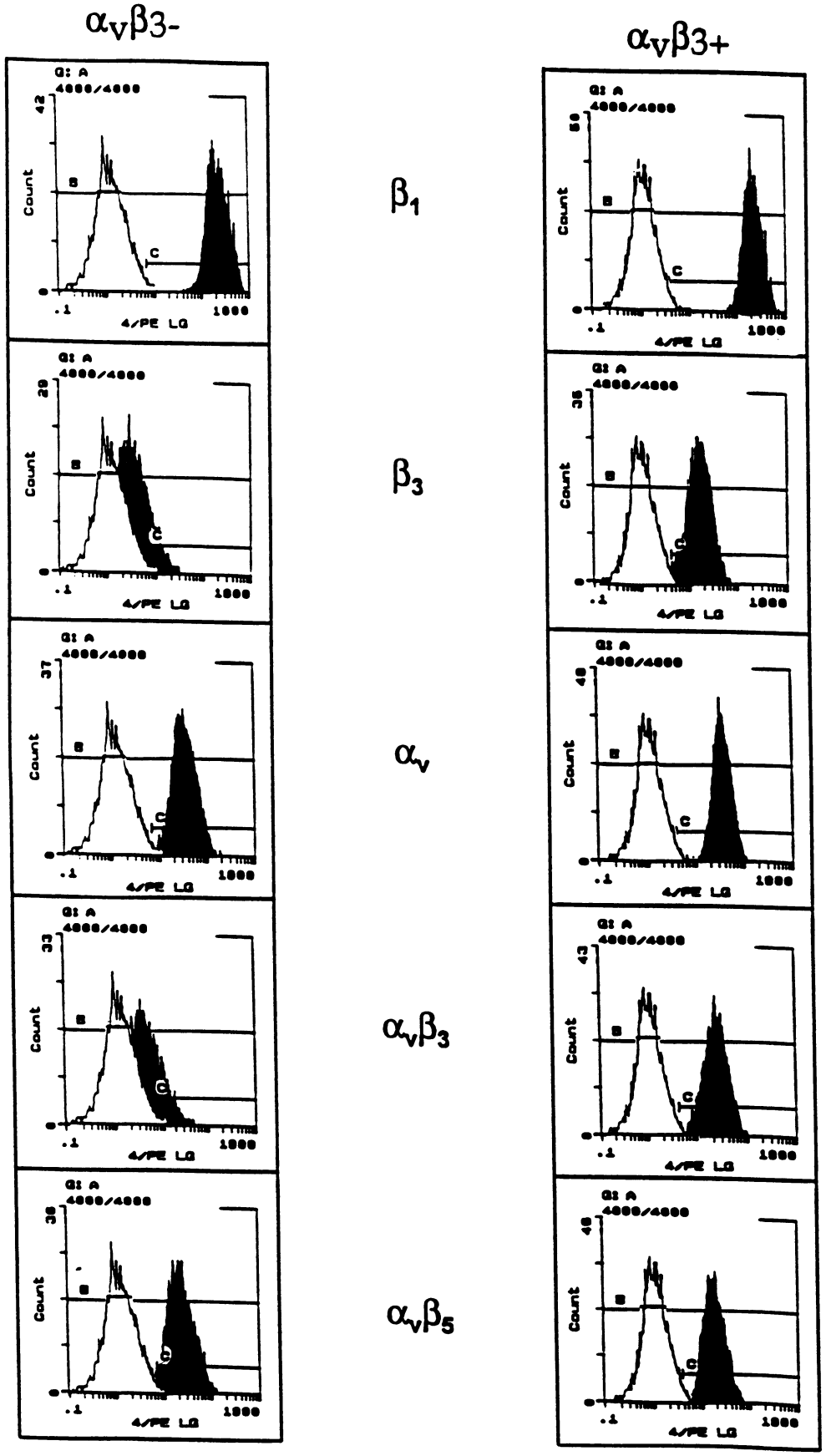

Figure 1. Expression of $\alpha_v\beta_3$ integrin is variable in two human smooth muscle cell isolates. Smooth muscle cells were fixed and incubated with either no primary antibody (*white peaks*) or antibodies against the indicated integrins (*black peaks*). A fluorescent secondary antibody was applied and cells were analyzed by flow cytometry. The y-axes represent cell number, and the x-axes represent log fluorescent intensity. Antibodies used were clones P4C10 (β_1), SZ 21 and Y2/51 (β_3), LM142 and AMF/7 (α_v), LM609 ($\alpha_v\beta_3$), and P1F6 and P3G2 ($\alpha_v\beta_5$). Identical results were obtained with different antibodies directed against a particular integrin.

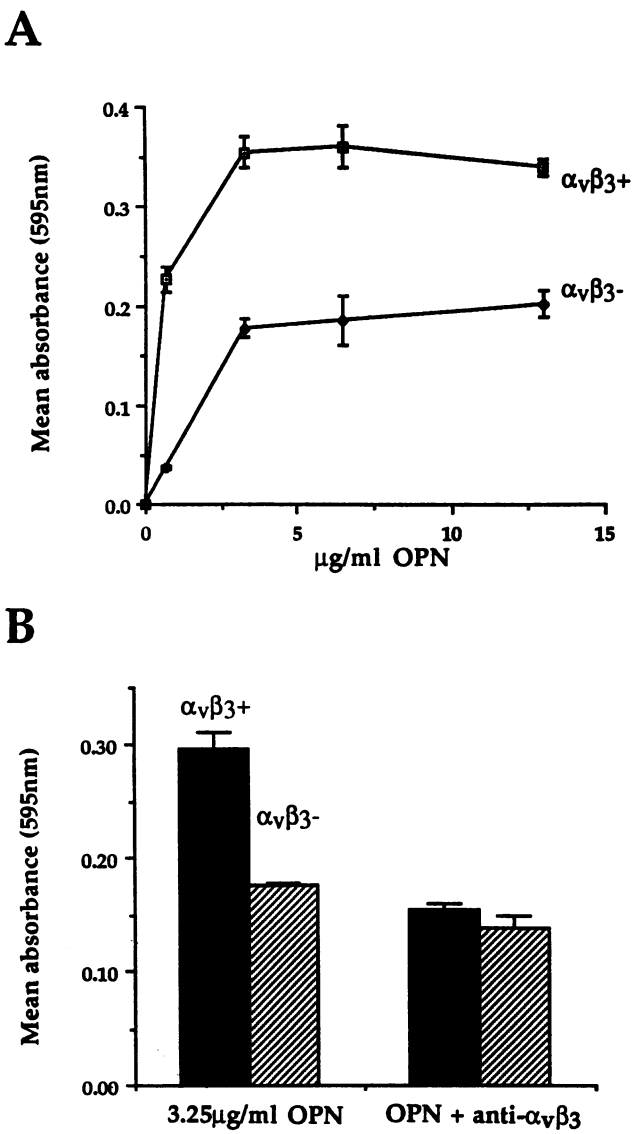

Figure 2. Both $\alpha_v\beta_3^-$ and $\alpha_v\beta_3^+$ smooth muscle cells adhere to an osteopontin (*OPN*) substrate. (*A*) Wells were coated overnight with 6.5 ng/ml to 13 μg/ml solutions of osteopontin. $\alpha_v\beta_3^-$ cells (*closed symbols*) and $\alpha_v\beta_3^+$ cells (*open symbols*) were plated at 30,000 cells/well and incubated for 60 min. Nonbound cells were rinsed off with PBS and adherent cells were quantitated as described. No cells bound to control wells coated with BSA alone (not shown). (*B*) Wells were precoated with a 3.25-μg/ml solution of osteopontin. $\alpha_v\beta_3^+$ cells (*solid bars*) and $\alpha_v\beta_3^-$ cells (*hatched bars*) were plated as above in wells either in the presence or absence of a 1:1,000 dilution of anti-$\alpha_v\beta_3$ ascites (LM609). Adhesion was quantitated after 60 min and expressed as means±SEM.

osteopontin adhesive receptors on smooth muscle cells. Other neutralizing antibodies including those directed against the α_2 and α_5 integrins did not affect adhesion to osteopontin in either cell strain, although they were able to block adhesion of smooth muscle cells to collagen and fibronectin, respectively (Fig. 4 *C*). We noted a small but statistically insignificant decrease in smooth muscle cell adhesion to osteopontin in the presence of anti-α_3 antibodies (Fig. 4, *A*, and *B*). These anti-α subunit antibodies were chosen since our flow cytometry results demonstrated that both smooth muscle cell strains contained α_2, α_3, α_5, and α_v but lacked detectable levels of α_4 or α_6 (Fig. 1 and reference 24). Antiosteopontin antibody OP199 effectively blocked adhesion of smooth muscle cells to the osteopontin substrate, thus assuring that the observed effects were osteopontin-specific (Fig. 4 *A*). In addition, ascite fluid containing an irrelevant isotype matched (IgG_1) antibody directed against the cell adhesion region of fibronectin was used as a negative control. Adhesion of both cell strains to osteopontin was not significantly affected by this antibody ($\alpha_v\beta_3^+$ cells exhibited 101% adhesion and $\alpha_v\beta_3^-$ cells exhibited 94% adhesion in the presence of antifibronectin compared with adhesion on osteopontin alone).

$\alpha_v\beta_1$ is the β_1-containing osteopontin receptor. To determine which α subunit forms a functional osteopontin receptor with β_1 in smooth muscle cells, three approaches were taken. First, we took advantage of a cyclic peptide that shows specificity for vitronectin receptors containing the α_v subunit Gly-Pen-Gly-Arg-Gly-Asp-Ser-Pro-Cys-Ala, (PenRGD, where Pen is penicillamine) (22, 23) to test if this blocked $\alpha_v\beta_3^-$ cell adhesion to osteopontin. Fig. 5 *A* shows that the PenRGD peptide inhibited smooth muscle cell adhesion to osteopontin in a dose-dependent manner without affecting adhesion to fibronectin, suggesting that these cells may use similar receptors to bind osteopontin and vitronectin. This effect was specific since an RGE-containing hexamer had negligible effect (Fig. 5, *inset*). Secondly, an antibody directed to α_v (L230) has recently been characterized to functionally neutralize adhesion mediated by $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ integrins, but not β_1 containing collagen receptors (21). We used this antibody to test if neutralizing α_v could abolish adhesion to osteopontin. Fig. 5 *B* demonstrates that neutralizing the α_v subunit completely eliminated adhesion of both isolates of smooth muscle cells to osteopontin, indicating that all osteopontin adhesion could be accounted for by α_v-containing integrins. Neutralization by this antibody was specific since adhesion of smooth muscle cells to the same molar concentration of fibronectin was not altered by equivalent concentrations of this antibody (adhesion to fibronectin, mean absorbance = 0.3±0.028 and adhesion in the presence of anti-α_v antibody, mean absorbance = 0.28±0.016). Lastly, we determined if the $\alpha_v\beta_1$ complex was present in smooth muscle cells. The integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are detectable on the surface of smooth muscle cells (Fig. 1), however no antibody is currently available that specifically recognizes $\alpha_v\beta_1$. To determine if $\alpha_v\beta_1$ forms a heterodimeric receptor in smooth muscle cells, cell lysates were immunoprecipitated with anti-β_1 antibody, and Western analysis was performed on the dissociated complexes using an anti-α_v antibody (Fig. 6). In two different human smooth muscle strains, immunoprecipitation with anti-β_1 antibody precipitated an $\alpha_v\beta_1$-containing complex, demonstrating the formation of this heterodimer in smooth muscle cells.

β_1 integrin on smooth muscle cells functions primarily as an osteopontin but not vitronectin receptor. The integrins we have implicated as smooth muscle cell osteopontin receptors, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_1$, have been previously shown to interact with vitronectin (25–30). Because of this potential overlap in osteopontin and vitronectin receptors, we sought to determine if smooth muscle cells use $\alpha_v\beta_1$ and $\alpha_v\beta_5$ to adhere to both proteins. As shown in Fig. 7, although anti-$\alpha_v\beta_5$ and anti-β_1

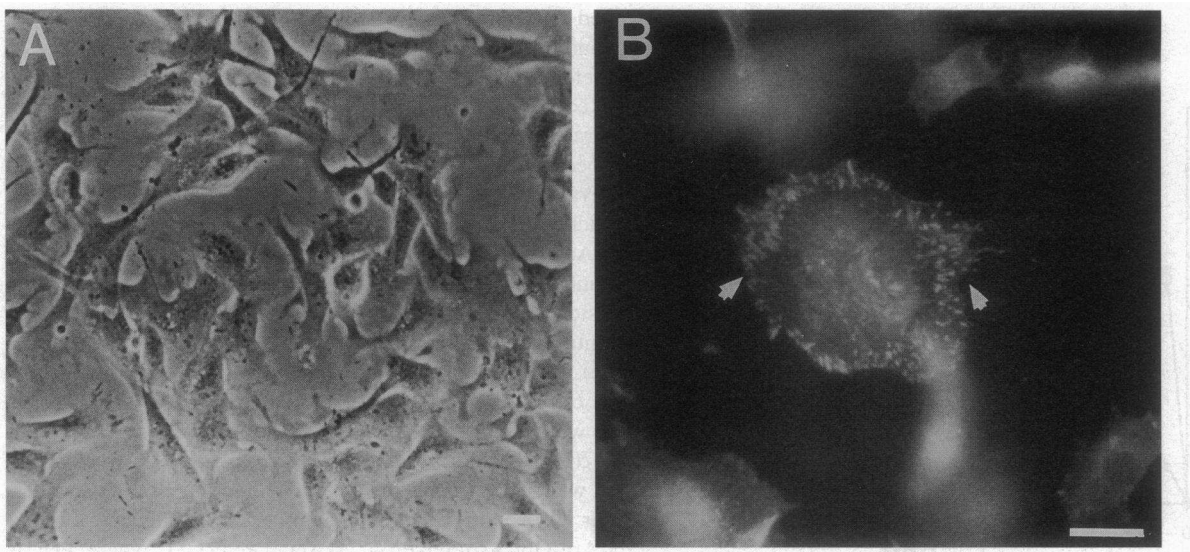

Figure 3. Adhesion of $\alpha_v\beta_3^-$ cells on osteopontin is accompanied by cell spreading and focal contact formation. (*A*) $\alpha_v\beta_3^-$ cells were plated in wells precoated with a 3.25-μg/ml solution of osteopontin and allowed to incubate for 60 min. The phase photograph was taken after nonadherent cells were washed off. Bar = 20 μm. (*B*) $\alpha_v\beta_3^-$ cells were plated in wells precoated with a 3.25-μg/ml solution of osteopontin, rinsed, and fixed after 60 min. Cells were stained with antivinculin, followed by a fluorescein-conjugated rabbit anti–mouse IgG. Vinculin localizes to focal contacts (*white arrows*). Bar = 20 μm. Original magnification of *A* was 200 and *B* was 400.

antibodies inhibited $\alpha_v\beta_3^-$ smooth muscle cell adhesion to osteopontin, only anti-$\alpha_v\beta_5$ blocked adhesion to vitronectin, and anti-β_1 had no effect on cell adhesion to vitronectin, even in combination with anti-$\alpha_v\beta_5$. Thus in $\alpha_v\beta_3^-$ smooth muscle cells, it appears that $\alpha_v\beta_1$ mediates adhesion to osteopontin while β_1 integrins do not interact with vitronectin to promote adhesion. In addition, the majority of smooth muscle cell adhesion to fibronectin appears to be via the α_5 integrin (Fig. 4 *C*), which does not contribute to adhesion on osteopontin (Fig. 4 *A*). Thus, $\alpha_v\beta_1$ on smooth muscle cells preferentially interacts with osteopontin and not with vitronectin or fibronectin.

Differences in migration of $\alpha_v\beta_3^-$ and $\alpha_v\beta_3^+$ cells to osteopontin. $\alpha_v\beta_3^+$ cells and $\alpha_v\beta_3^-$ cells were tested in a Boyden-type chamber for migration toward osteopontin (Fig. 8 *A*) on a fibronectin-coated filter. $\alpha_v\beta_3^+$ cell migration was observed in response to osteopontin after 5 h, with significant increases in migration seen at 5 μg/ml and maximal effects at 20 μg/ml in the lower chamber. In sharp contrast, $\alpha_v\beta_3^-$ cells did not migrate to any concentration of osteopontin tested. $\alpha_v\beta_3^-$ cells were not merely slow to respond to osteopontin, since no migration above background was seen even when cells were allowed to migrate for up to 12 h. Migration assays were performed at the shorter time point (5 h) so that confounding factors including cell replication, loss of concentration gradients, and synthesis and secretion of cellular proteins were minimized. To rule out the possibility that $\alpha_v\beta_3^-$ cells were not migrating because they were less adhesive to the fibronectin-coated filter, the ability of both cell types to attach to fibronectin was determined (Fig. 8 *B*). There was no difference in adhesion of either cell strain to fibronectin. Despite the striking difference in the ability of these cells to migrate toward osteopontin, $\alpha_v\beta_3^-$ cells were not generally deficient in migratory capacity, since they showed an average of an eightfold increase in migration toward 10% fetal bovine serum compared with an average sevenfold increase in $\alpha_v\beta_3^+$ cell migration to 10% serum from a similar baseline level (data not shown). In addition, smooth muscle cells deficient in $\alpha_v\beta_3$ have also been shown to migrate on collagen-coated surfaces toward platelet-derived growth factor BB and insulin-like growth factor-1 (24). The $\alpha_v\beta_3^-$ cells were also stimulated to migrate significantly with a vitronectin or fibronectin stimulus in the lower chamber (Fig. 8 *C*). However, the migration of $\alpha_v\beta_3^-$ cells to vitronectin and fibronectin remained approximately sixfold lower than $\alpha_v\beta_3^+$ cell migration to osteopontin or vitronectin (Fig. 8 *D*). Finally, an anti-$\alpha_v\beta_3$ antibody (LM609) was used to verify the role of this integrin in mediating $\alpha_v\beta_3^+$ cell migration toward osteopontin. As shown in Fig. 8 *D*, specific migration of $\alpha_v\beta_3^+$ cells was inhibited in a dose-dependent manner in the presence of anti-$\alpha_v\beta_3$ antibody (LM609) and was eliminated at antibody concentrations of 10 μg/ml or above. Of interest, the anti-$\alpha_v\beta_3$ antibody was not able to inhibit cell migration to vitronectin (Fig. 8 *D*), consistent with the finding that $\alpha_v\beta_3^-$ cells were able to migrate toward a vitronectin gradient (Fig. 8 *C*). No effect on basal migration (BSA alone) was seen when cells were treated by LM609 (Fig. 8 *D*).

Distribution of integrins on cells attached to osteopontin. Functional interaction of substrates with integrin receptors often leads to organization of those receptors into focal contacts. To determine the localization of $\alpha_v\beta_5$ and β_1 integrins in $\alpha_v\beta_3^-$ cells, cells were allowed to spread on an osteopontin substrate, and $\alpha_v\beta_5$ and β_1 were detected by immunofluorescence. Localization of these integrins was compared with the distribution of $\alpha_v\beta_3$. Cells adherent to osteopontin localize $\alpha_v\beta_3$ in focal contact regions (Fig. 9, *B* and *C*) in both endothelial cells and human smooth muscle cells, in agreement with our previous report (4). In contrast, $\alpha_v\beta_5$ remained diffuse on the cell surface (Fig. 9 *D*), while β_1 localized to the periphery at cell borders of cells adherent to osteopontin (Fig. 9 *E*). The immunofluorescent patterns observed were specific since minimal nonspecific cell binding of the fluorochrome was detected when no primary

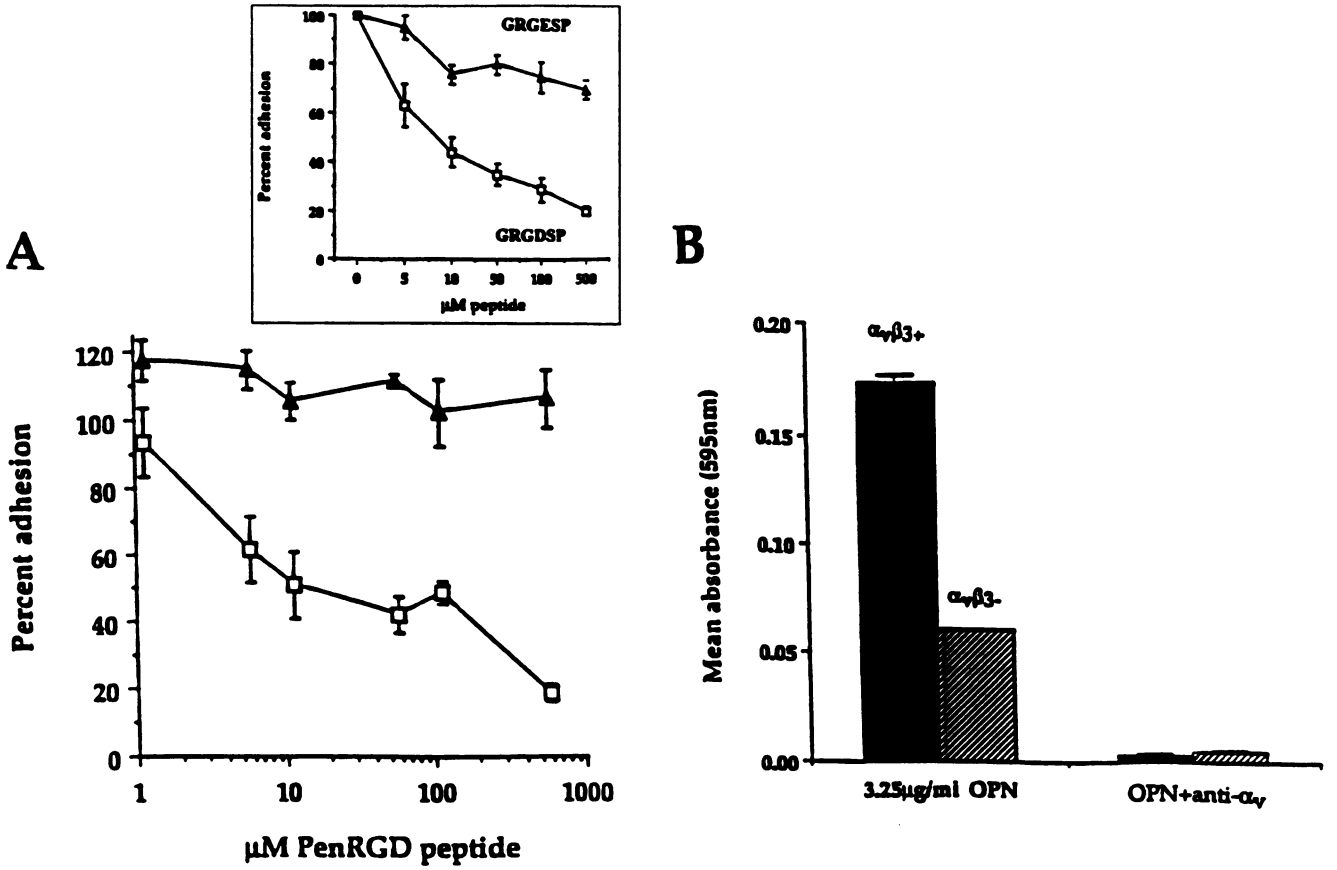

Figure 5. Adhesion is blocked by a peptide specific to vitronectin receptors and an anti-α_v neutralizing antibody. (*A*) $\alpha_v\beta_3^-$ cells were plated in wells precoated with either 50 nM solutions of osteopontin (3.25 μg/ml, *open squares*) or fibronectin (11 μg/ml, *dark triangles*) with increasing amounts of the cyclic peptide PenRGD. Cell adhesion was quantitated after 60 min and calculated as the percent adhesion of control, where no peptide was added. (*Inset*) Cells were plated on osteopontin in the same conditions as in *A* except in the presence of either GRGDSP or GRGESP peptides. (*B*) Wells were precoated with a 3.25-μg/ml solution of osteopontin overnight. $\alpha_v\beta_3^+$ cells (*solid bars*) and $\alpha_v\beta_3^-$ cells (*hatched bars*) were plated in wells either in the presence or absence of L230 antibody (final concentration of 5%) previously characterized as specific to the α_v subunit. Assay was quantitated after 60 min, and data are expressed as means±SEM.

antibody (Fig. 9 *A*) or a isotype-matched control antibody was used.

Discussion

Human vascular smooth muscle cell strains containing either high or very low levels of $\alpha_v\beta_3$ integrin were identified using immunofluorescent flow cytometric analysis. Using these cells, we have characterized osteopontin receptors and their functions on smooth muscle cells. Three major conclusions can be drawn from our results. First, osteopontin can interact with $\alpha_v\beta_5$ and $\alpha_v\beta_1$ integrin in addition to $\alpha_v\beta_3$ on smooth muscle cells. Second, cell surface $\alpha_v\beta_3$ appears necessary for vascular smooth muscle cell migration to osteopontin and affects adhesive capacity on an osteopontin substrate. Finally, β_1 integrins on smooth muscle cells mediate adhesion to osteopontin but not vitronectin. These data strongly argue that at least two different functions of osteopontin, adhesion and migration, depend upon interactions of this protein with distinct receptors.

Osteopontin interacts with multiple cell surface receptors. Previous studies demonstrated that osteopontin interacts with the $\alpha_v\beta_3$ integrin in an RGD-dependent manner (10, 11, 31). Likewise, we determined recently that $\alpha_v\beta_3$ plays a role in endothelial and smooth muscle cell adhesion to osteopontin (4). However, interaction with $\alpha_v\beta_3$ alone could not account for all vascular cell adhesion to osteopontin. Therefore, in this report, two human smooth muscle cell strains that varied in their relative levels of $\alpha_v\beta_3$ integrin were used to identify additional osteopontin receptors. Our results indicate that the integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_1$ function as osteopontin adhesive receptors in human smooth muscle cells. This is a novel description of $\alpha_v\beta_5$ and $\alpha_v\beta_1$ integrins as osteopontin receptors. There was also a slight but consistent decrease in cell adhesion in the presence of anti-α_3 antibody, suggesting that an α_3-containing integrin may also contribute to the formation or stabilization of these adhesive interactions. However, the ability of a neutralizing α_v antibody (L230) (21) to completely eliminate adhesion to osteopontin is strong evidence that α_v can dimerize with three β subunits, β_1, β_3, and β_5, to form the major osteopontin adhesive receptors on smooth muscle cells. Although smooth muscle cell adhesion to osteopontin can be blocked using RGD-containing peptides, our studies do not rule out the possibility that α_v-containing receptors may also interact with other regions of osteopontin. This possibility is supported by the finding by

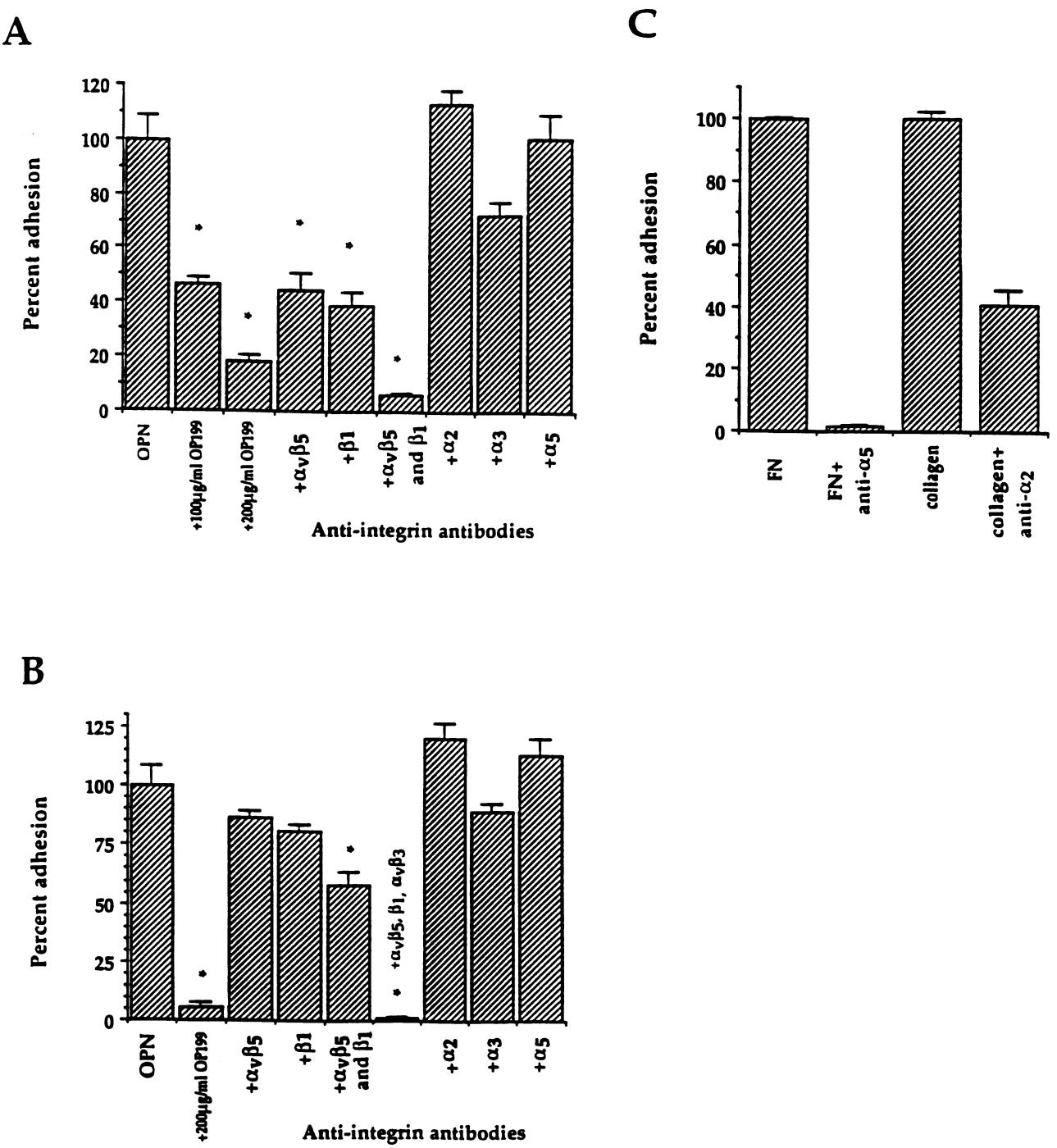

Figure 4. Multiple integrins interact with osteopontin. $\alpha_v\beta_3^-$ cells (*A*) and $\alpha_v\beta_3^+$ cells (*B*) were plated (30,000 cells/well) into wells previously coated with a 3.25-μg/ml osteopontin solution, in the presence or absence of antibodies against osteopontin (OP199), $\alpha_v\beta_5$ (P1F5), β_1 (P4C10), α_2 (P1E6), α_3 (P1B5), and α_5 (P1D6). OP199 was purified IgG, and the others were ascites used at a 1:500 dilution. Assay time was 60 min. Asterisks indicate significance at 95% with Scheffé's F test for multiple comparisons. (*C*) $\alpha_v\beta_3^-$ cells were plated into wells precoated with a 2.2-μg/ml solution of fibronectin (*FN*) or 0.3-μg/ml solution of collagen with or without antibodies against α_2 (P1E6) and α_5 (P1D6) at a 1:500 dilution of ascites. Data are expressed as means±SEM.

van Dijk et al. (32) that a proteolytic fragment of osteopontin lacking the RGD sequence was able to support adhesion of human gingival fibroblasts.

Interaction of osteopontin with different integrins has distinct functional consequences. Comparison of smooth muscle cells with variable levels of surface $\alpha_v\beta_3$ enabled us to note differences in both adhesion and migration of these two cell populations to osteopontin. All three integrins, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and

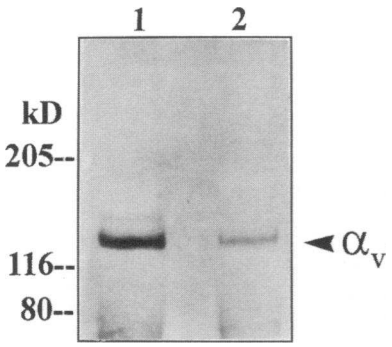

Figure 6. The $\alpha_v\beta_1$ complex is present in smooth muscle cells. (*Lane 1*) retrovirally transfected adult smooth muscle cells (2.2 million total) and (*lane 2*) $\alpha_v\beta_3^-$ cells (1.2 million total) were lysed and cell extracts were immunoprecipitated with anti-β_1 (P4C10) followed by rabbit anti-goat IgG and protein A conjugates. Immune complexes were dissociated, run on a 5% SDS-PAGE gel, and transferred to a PVDF membrane. Anti-α_v antibody was used in Western blot analysis to establish that $\alpha_v\beta_1$ was present as a complex in both cell strains.

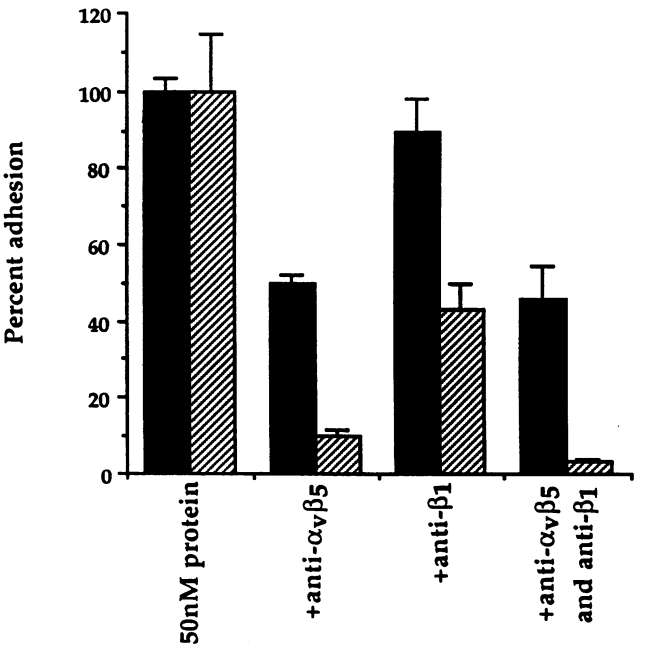

Figure 7. The β_1 integrin on smooth muscle cells acts as an osteopontin not a vitronectin receptor. Wells were precoated with 50 nM solutions of either vitronectin (3.5 μg/ml, *solid bars*) or osteopontin (3.25 μg/ml, *hatched bars*). $\alpha_v\beta_3^-$ cells were plated in the absence (no antibody) or presence of a 1:500 dilution of ascites of anti-$\alpha_v\beta_5$ (P1F6), anti-β_1 (P4C10), or both. Adhesion was quantitated after 60 min, and data are expressed as means±SEM.

$\alpha_v\beta_5$, act as osteopontin adhesive receptors. However, adhesion of the $\alpha_v\beta_3$-deficient strain to osteopontin was compromised. This difference in adhesion of the two cell strains was not noted on a fibronectin substrate, where equal adhesion was obtained. A particularly exciting finding was the contrasting migratory potential of these two cell strains. Cells deficient in $\alpha_v\beta_3$ were unable to migrate toward an osteopontin gradient, in contrast to $\alpha_v\beta_3^+$ cells which exhibited a strong migratory response that was eliminated by an anti-$\alpha_v\beta_3$ neutralizing antibody. $\alpha_v\beta_5$ and $\alpha_v\beta_1$ are apparently not able to mediate migration toward osteopontin. It is interesting to speculate that the $\alpha_v\beta_3$ integrin may be generally important for smooth muscle cell migration toward some stimuli and matrices. This is supported by our observations that $\alpha_v\beta_3$-deficient cells were stimulated to migrate to vitronectin, fibronectin, and serum, but relative numbers of migrated cells were 6–10-fold lower than their $\alpha_v\beta_3$-containing counterparts. Work by others has suggested that $\alpha_v\beta_3$-containing smooth muscle cells from sheep ductus also require $\alpha_v\beta_3$ integrin for migration on various matrix substrates (33).

Our results support the hypothesis that osteopontin mediates various functions by selective interaction with receptors. This idea has a growing body of support from studies addressing interactions of vitronectin and fibronectin through their multiple receptors. Both $\alpha_v\beta_5$ and $\alpha_v\beta_3$ have been well established as adhesive vitronectin receptors, although only $\alpha_v\beta_3$ localized to focal contacts and led to cell spreading and migration toward vitronectin (14, 34). Cell migration initiated through occupancy of either β_1 or β_3 integrins was later shown to correspond to different signaling mechanisms (35). Conversely, it has been reported recently that the $\alpha_v\beta_5$ but not $\alpha_v\beta_3$ vitronectin receptor was involved in internalization and degradation of vitronectin in the matrix (36). Likewise for fibronectin, functional differences have emerged in the signaling generated by receptor occupancy of either $\alpha_v\beta_1$ or $\alpha_5\beta_1$. In this case, both integrins functioned as adhesive receptors, although only $\alpha_5\beta_1$ supported fibronectin matrix assembly and migration toward fibronectin (29). Our findings support the hypothesis that, like vitronectin and fibronectin, osteopontin has diverse functions that are mediated via distinct receptors.

β_1 integrins on smooth muscle cells promote adhesion to osteopontin but not vitronectin. The complement of osteopontin receptors observed in our study is similar to the integrins that have been reported to interact with vitronectin, another smooth muscle adhesive protein with chemotactic properties (37). The integrin $\alpha_v\beta_5$ interacts with vitronectin (27, 37) and $\alpha_v\beta_1$ has been shown in some cell lines to bind vitronectin (28), fibronectin (29, 30, 32), and possibly type I collagen (38). Our data suggest that, in smooth muscle cells, β_1 integrin does not play a major role in vitronectin adhesion, but $\alpha_v\beta_1$ is involved in adhesion to osteopontin. In addition, recent reports of osteopontin receptors on osteoclasts and chondrocytes described $\alpha_v\beta_3$ as mediating attachment to osteopontin without the contribution of β_1 receptors (11, 31, 39), which were also present on the cell surface. While the mechanisms underlying the apparent cell type specificity of osteopontin and vitronectin interaction with the β_1 integrin were not addressed by our studies, it is becoming increasingly apparent that functional integrin–ligand interactions depend on multiple factors, including integrin activation state (40) and cell type. It will be of interest in the future to determine whether the state of β_1 activation in smooth muscle accounts for its observed osteopontin specificity.

Significance. In vivo, osteopontin is though to play an important developmental and maintenance role in a number of tissues including bone. Studies have clearly indicated that osteopontin is required for adhesion and signaling of osteoclasts during bone resorption and have implicated $\alpha_v\beta_3$ as the receptor involved in this process (10, 11). Yet in a subset of patients with Glanzmann's thrombasthenia, an autosomal recessive hemorrhagic disease caused in some cases by a defective β_3 gene (41), no bone defects are present that would be predicted if $\alpha_v\beta_3$ were the only osteopontin receptor. The presence of multiple

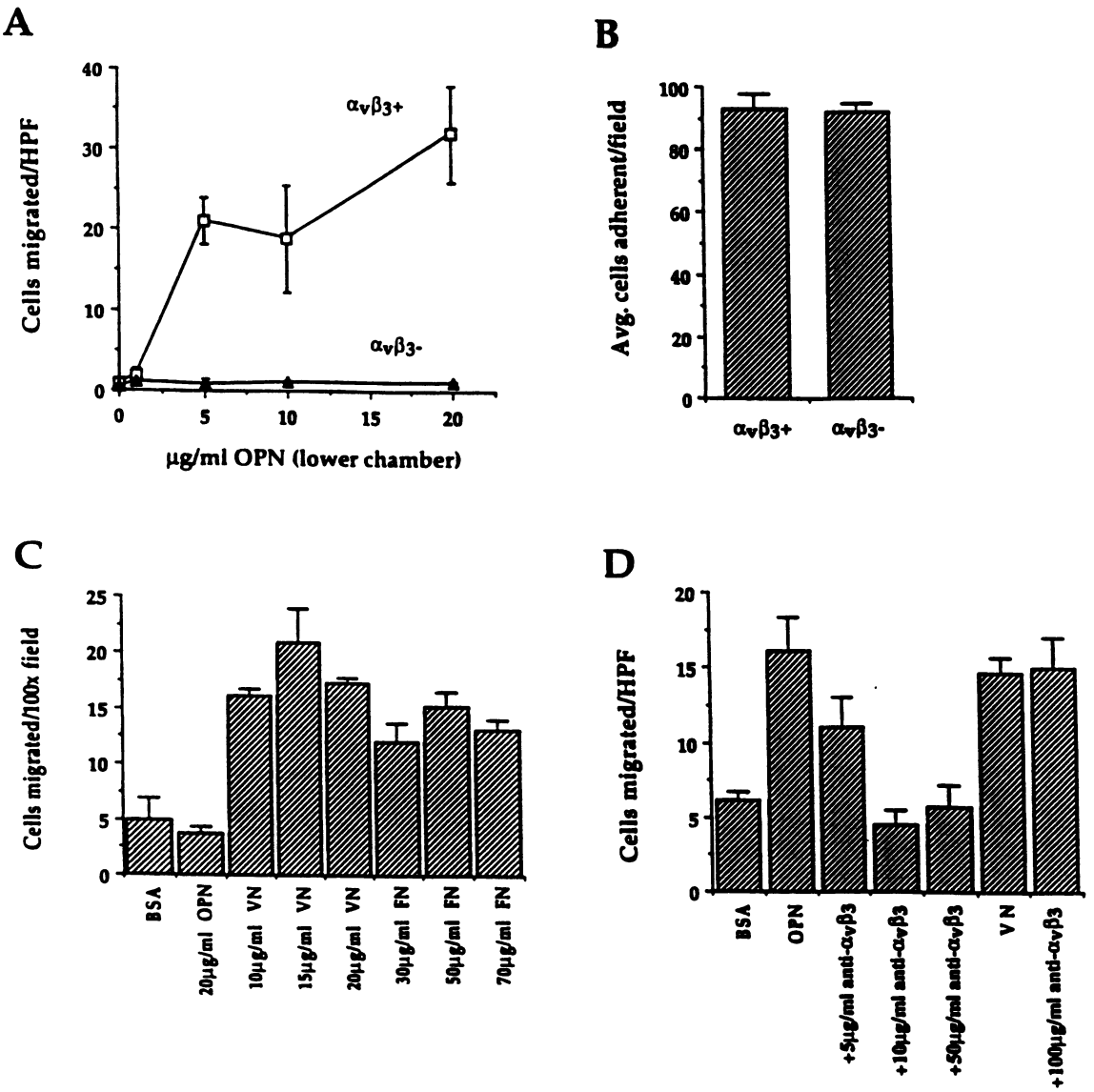

Figure 8. $\alpha_v\beta_3^+$ but not $\alpha_v\beta_3^-$ smooth muscle cells migrate toward osteopontin. (*A*) $\alpha_v\beta_3^+$ cells (*open squares*) and $\alpha_v\beta_3^-$ cells (*dark triangles*) were plated in a Boyden-type chamber assay and tested for chemotaxis toward increasing concentrations of osteopontin in the lower chambers through a fibronectin-coated filter. Cells that had migrated to the lower chamber through the porous filter were quantitated after 5 h by cell counts of HPF (×400). (*B*) $\alpha_v\beta_3^+$ cells and $\alpha_v\beta_3^-$ cells were tested for their ability to adhere to wells precoated with 100 μg/ml fibronectin. 20,000 cells were plated in each well and were allowed to adhere for 60 min. Adhesion was quantitated by cell counts of ×200 fields. (*C*) $\alpha_v\beta_3^-$ cells were tested for migration as above to osteopontin (*OPN*), vitronectin (*VN*), and fibronectin (*FN*). Concentrations were chosen to represent equimolar amounts of VN and FN. Migration was quantitated after 5 h by cell counts of ×100 fields instead of ×400 fields due to lower cell migration of this smooth muscle cell strain. (*D*) $\alpha_v\beta_3^-$ cells were tested for migration toward 20 μg/ml of either OPN or VN in the lower chamber in the presence of increasing concentrations of anti-$\alpha_v\beta_3$ antibody (LM609). The assay was stopped after 5 h and cell counts were averaged per HPF (×400). Data are expressed as means±SEM.

osteopontin receptors capable of interacting with osteopontin may help explain this observation and predicts that in these patients $\alpha_v\beta_5$ or $\alpha_v\beta_1$ integrins might functionally replace $\alpha_v\beta_3$.

In the vascular system, where we first identified osteopontin as a product of injured smooth muscle cells during neointima formation, recent data have shown that PenRGD peptide significantly inhibited neointimal lesion development in a rabbit balloon catheter injury model (42). The same PenRGD peptide antagonized smooth muscle cell interaction with osteopontin (Fig. 5 *A*). Our data suggest that one of the processes that may be inhibited in the rabbit injury model is osteopontin interaction with its receptors. It will be interesting to determine if the effects of PenRGD on neointimal formation represent an inhibition of osteopontin functions. There have been limited studies on integrin expression in vivo in vessels, although in vitro we have seen β_3 mRNA expression in several fetal human smooth muscle cell samples. In addition, the growth factors PDGF and TGFβ also have been shown to induce β_3 expression in rabbit and bovine

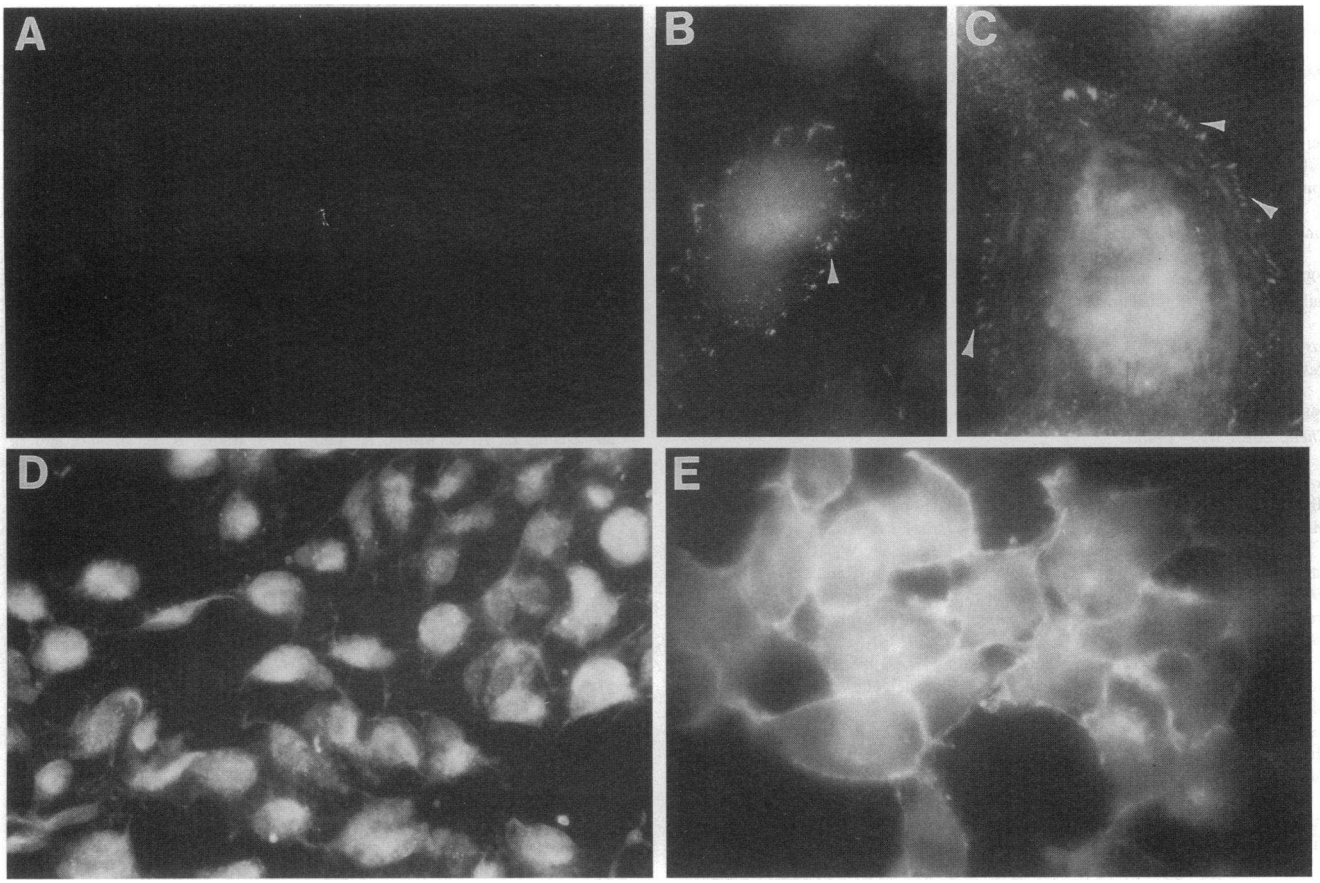

Figure 9. Integrins $\alpha_v\beta_5$, β_1, and $\alpha_v\beta_3$ localize differently in cells adherent to osteopontin. Slides were precoated with 3.25 μg/ml osteopontin, and $\alpha_v\beta_3^+$ cells (*A, C*), $\alpha_v\beta_3^-$ cells (*D, E*), or endothelial cells (*B*) were plated and incubated for 60 min. Cells were fixed, permeabilized, and stained with (*A*) no primary antibody, (*B*) anti-$\alpha_v\beta_3$ (LM609) on adherent endothelial cells, (*C*) anti-$\alpha_v\beta_3$ (LM609), (*D*) anti-$\alpha_v\beta_5$ (P3G2), or (*E*) anti-β_1 (P4C10) for 60 min. A fluorescein-conjugated anti-mouse secondary was added for an additional 60 min. White arrows in *B* and *C* show punctate localization of $\alpha_v\beta_3$ in focal adhesion areas. Original magnifications for all were 400, except *E,* which was 1,000.

smooth muscle cells (43, 44). We would predict a coordinate regulation of osteopontin and its receptors after vascular injury, when cells are induced to proliferate and migrate. Secondly, several groups have described the elevated production of osteopontin in atherosclerotic lesions (1–3). Production of osteopontin by both smooth muscle cells and foam cells correlated both with degree of atheroma as well as calcification. Our understanding of osteopontin's role in lesion development will be aided by our knowledge of the receptors that are potentially involved.

In summary, we have identified several different receptors capable of interacting with osteopontin on vascular smooth muscle cells: $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_1$ integrin. Furthermore, the adhesive and migratory effects of osteopontin on these cells appear to be mediated via distinct receptors. Thus, one mechanism explaining the functional diversity previously observed for osteopontin is its capacity to interact with three integrins, each potentially regulating a discrete function. We expect the expression and regulation of these integrins as well as osteopontin will be key factors controlling cellular responses to this multifunctional, cell-interactive glycoprotein.

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