The synergistic activity of $\alpha_v\beta_3$ integrin and PDGF receptor increases cell migration

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SUMMARY

Integrins and growth factor receptors act synergistically to modulate cellular functions. The $\alpha_v\beta_3$ integrin and the platelet-derived growth factor receptor have both been shown to play a positive role in cell migration. We show here that a platelet derived growth factor-BB gradient stimulated migration of rat microvascular endothelial cells on vitronectin (9.2-fold increase compared to resting cells) in a $\alpha_v\beta_3$ and RGD-dependent manner. In contrast, this response was not observed on a $\beta_1$ integrin ligand, laminin; background levels of migration, in response to a platelet derived growth factor-BB gradient, were observed on this substrate or on bovine serum albumin (2.4- or 2.0-fold, respectively). Comparable results were obtained using NIH-3T3 cells. Platelet derived growth factor-BB did not change the cells' ability to adhere to vitronectin, nor did it stimulate a further increase in proliferation on vitronectin versus laminin. In addition, platelet derived growth factor-BB stimulation of NIH-3T3 cells did not alter the ability of $\alpha_v\beta_3$ to bind RGD immobilized on Sepharose. The $\alpha_v\beta_3$ integrin and the platelet derived growth factor receptor-$\beta$ associate in both microvascular endothelial cells and NIH-3T3 cells, since they coprecipitated using two different antibodies to either $\alpha_v\beta_3$ or to the platelet derived growth factor receptor-$\beta$. In contrast, $\beta_1$ integrins did not coprecipitate with the platelet derived growth factor receptor-$\beta$. These results point to a novel pathway, mediated by the synergistic activity of $\alpha_v\beta_3$ and the platelet derived growth factor receptor-$\beta$, that regulates cell migration and, therefore, might play a role during neovessel formation and tissue infiltration.

Key words: Integrin, Platelet derived growth factor receptor, Migration

INTRODUCTION

Cell-extracellular matrix interactions are predominantly mediated by integrins, cell surface receptors which exist as heterodimers of noncovalently associated $\alpha$ and $\beta$ subunits (Hynes, 1992). Integrins regulate several cellular functions including cell migration, growth and differentiation (Damsky and Werb, 1992; Juliano, 1996; Ruoslahti and Reed, 1994). A subgroup of $\alpha_v$-containing integrins, specifically the vitronectin receptor $\alpha_v\beta_3$ (Felding-Habermann and Cheresh, 1993), has been shown to modulate endothelial cell migration (Leavesley et al., 1993) on vitronectin-coated substrates. Vitronectin is an extracellular matrix protein found, in vivo, in arterial blood vessels in areas of pericytic matrices (Preissner, 1991). The $\alpha_v\beta_3$ integrin is expressed by both microvascular (Klein et al., 1993) and large vessel endothelial cells (Cheng and Kramer, 1989; Fitzgerald et al., 1987; Languino et al., 1989; Preissner et al., 1988). The $\alpha_v\beta_3$ integrin has been shown to have a key role in angiogenesis (Brooks et al., 1994a, 1995; Friedlander et al., 1995; Gamble et al., 1993), a process that requires changes in endothelial cell migration, proliferation, cell-cell contact, cell-matrix interactions and ultimately, lumen formation (Madri et al., 1996; Vernon and Sage, 1995).

Synergistic effects of growth factors and integrins in modulating cell functions have been described (Schwartz et al., 1995). The cascade of intracellular signaling events triggered by platelet derived growth factor (PDGF; Claesson-Welsh, 1996) results in enhanced cell migration and proliferation via actin cytoskeletal reorganization, disruption of focal contacts and redistribution of tyrosine phosphorylated proteins (Pascuale et al., 1988; Rankin and Rozengurt, 1994; Ridley and Hall, 1992). Specifically, PDGF-BB stimulation increases chemotaxis of rat brain capillary endothelial cells (Risau et al., 1992). The presence of the PDGF receptor-$\beta$ (PDGFR$\beta$) has been extensively documented in microvascular endothelial cells (Bar et al., 1989; Beitz et al., 1991; Hermansson et al., 1988; Marx et al., 1994; Plate et al., 1992; Smits et al., 1989; Streuten et al., 1989) and the role of both PDGF-BB and -AA in inducing formation of new vessels has been shown (Risau et al., 1992; Nicosia et al., 1994). However, a potential synergistic effect of both $\alpha_v\beta_3$ and PDGFR$\beta$ engagement on microvascular endothelial cell migration has not been studied.
We have examined the synergistic activity of $\alpha_v\beta_3$ and the PDGFR$\beta$ both in rat microvascular endothelial cells (RFCs) and, in order to broaden the scope of our findings, in a different cell type, NIH-3T3 cells. We show here that PDGF-BB stimulation of both RFCs and NIH-3T3 cells resulted in an increase in cell migration on vitronectin, without affecting either proliferation or adhesion to this substrate or $\alpha_v\beta_3$ binding to RGD. PDGFF-BB caused loss of $\alpha_v$ focal contact localization in a cell type specific manner. In addition, we demonstrate that $\alpha_v\beta_3$, but not $\alpha_v\beta_1$, associates with the PDGFR$\beta$. These results suggest that $\alpha_v\beta_3$ integrin and PDGF-BB may act synergistically in vivo to promote neovessel formation and tissue infiltration.

MATERIALS AND METHODS

Cell culture
NIH-3T3 cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% newborn calf serum (NCS, Life Technologies, Inc.), 100 units/ml penicillin, 0.1 mg/ml streptomycin (Life Technologies, Inc.), 0.292 mg/ml L-glutamine (Life Technologies, Inc.), 1 mM sodium pyruvate (Life Technologies, Inc.). RFCs isolated from sodium pyruvate (Life Technologies, Inc.), and 1 mM sodium pyruvate (Life Technologies, Inc.). RFCs isolated from mouse laminin or human vitronectin (Life Technologies, Inc.) or 1% bovine serum albumin (BSA, Sigma) at either 37°C for one hour or 4°C overnight. For RFCs, human vitronectin or mouse laminin were used at 5 µg/ml or 3 µg/ml, respectively. In all of the experiments described, the concentration of vitronectin and laminin used for coating had been previously determined to generate comparable cell attachment to the inserts (data not shown). For NIH-3T3 cells, the filters were coated with either human vitronectin or rat laminin at a concentration of 3 µg/ml. The inserts were then blocked with 1% BSA in phosphate buffered saline (PBS) at 37°C for 30 minutes to one hour and subsequently with DMEM containing 0.1% BSA and 0.5% serum. Cells which had been serum starved overnight were detached using 0.05% trypsin, 0.53 mM EDTA (Life Technologies, Inc.), resuspended in DMEM containing 0.5% serum and then lysed in 10% SDS (American Bioanalytical, Natick, MA). Thymidine incorporation was determined by liquid scintillation counting. To quantitate adhesion to each substrate, attached cells, in control wells, were measured as described above. Proliferation assays for NIH-3T3 cells were performed as described for RFCs with the following modifications. The 96-well plates were coated with either human vitronectin (30 µg/ml) or mouse laminin (10 µg/ml), at either 37°C for one hour or 4°C overnight. The plates were washed two times with PBS and blocked with DMEM containing 1% BSA and 0.5% calf serum, at 37°C for 30 minutes to one hour. 1×10^4 RFCs were plated in DMEM containing 0.5% calf serum and incubated at 37°C. After 13.5 hours, PDGF-BB (3 ng/ml) stimulated or resting cells were incubated with 1 µCi [3H]thymidine per well (Amersham, Arlington Heights, IL) for an additional 24 hours at 37°C. The plates were washed two times with DMEM containing 0.5% calf serum and then lysed in 10% SDS (American Bioanalytical, Natick, MA). Thymidine incorporation was determined by liquid scintillation counting. To quantitate adhesion to each substrate, attached cells, in control wells, were measured as described above. Proliferation assays for NIH-3T3 cells were performed as described for RFCs with the following modifications. The 96-well plates were coated with either human vitronectin (3 µg/ml) or rat laminin (30 µg/ml). 2×10^4 NIH-3T3 cells in DMEM containing 0.5% NCS were plated in each well. After 36 hours, cells were either stimulated with 3 ng/ml PDGF-BB or left quiescent. Thymidine was added 12 hours after growth factor addition. Four hours later, the cells were washed and lysed as described above. Each experimental point was performed in duplicate.

Affinity chromatography
To purify $\alpha_v$ integrin complexes from NIH-3T3 cells, the following procedure was used. Serum starved, PDGF-BB stimulated (30 ng/ml at 37°C for 10 minutes) or resting NIH-3T3 cells were harvested with 4 mM EDTA (J. T. Baker, Phillipsburg, NJ) in PBS and solubilized in Trition-lysis buffer: 20 mM Tris-HCl, pH 8.0 (American Bioanalytical), 1% Triton X-100 (Sigma), 10% glycerol (J. T. Baker, Phillipsburg, NJ) in Triton-lysis buffer: 20 mM Tris-HCl, pH 8.0 (American Bioanalytical). Briefly, cells that were serum starved overnight were resuspended in DMEM containing 0.5% serum and either stimulated with 30 ng/ml PDGF-BB in suspension 10 minutes before seeding, or 10 minutes after seeding. PDGF-BB remained in the wells throughout the assay. Control cells were not stimulated with PDGF-BB. Cells were incubated at 37°C for either 90 (RFC) or 25 minutes (NIH-3T3), washed with PBS, fixed with 3% paraformaldehyde at 4°C and stained with 0.5% Crystal Violet at room temperature. Adhesion was quantitated by measuring the absorbance at 630 nm using a microtiter plate reader (ICN Titertek Multiskan Bichromatic, Irvine, CA). Each experimental point was performed in duplicate.

Proliferation assay
For RFCs, 96-well Linbrotitertek plates (Flow Laboratories, McLean, VA) were coated with either human vitronectin (30 µg/ml) or mouse laminin (10 µg/ml), at either 37°C for one hour or 4°C overnight. The wells were washed three times with PBS and blocked with DMEM containing 1% BSA and 0.5% calf serum, at 37°C for 30 minutes to one hour. 1×10^4 RFCs were plated in DMEM containing 0.5% calf serum and incubated at 37°C. After 13.5 hours, PDGF-BB (3 ng/ml) stimulated or resting cells were incubated with 1 µCi [3H]thymidine per well (Amersham, Arlington Heights, IL) for an additional 24 hours at 37°C. The plates were washed two times with DMEM containing 0.5% calf serum and then lysed in 10% SDS (American Bioanalytical, Natick, MA). Thymidine incorporation was determined by liquid scintillation counting. To quantitate adhesion to each substrate, attached cells, in control wells, were measured as described above. Proliferation assays for NIH-3T3 cells were performed as described for RFCs with the following modifications. The 96-well plates were coated with either human vitronectin (3 µg/ml) or rat laminin (30 µg/ml). 2×10^4 NIH-3T3 cells in DMEM containing 0.5% NCS were plated in each well. After 36 hours, cells were either stimulated with 3 ng/ml PDGF-BB or left quiescent. Thymidine was added 12 hours after growth factor addition. Four hours later, the cells were washed and lysed as described above. Each experimental point was performed in duplicate.

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Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) and blocked in 5% non-fat dry milk (Carnation, Glendale, CA), 0.2% NP-40 (Calbiochem) in TBS at 4°C. The membranes were incubated overnight at 4°C with a 1:500 dilution of rabbit antisera against the cytoplasmic domain of the human β3 integrin subunit (provided by Erkki Ruoslahti). Filters were washed with blocking buffer and then incubated with 125I-Protein-A (ICN) in blocking buffer at room temperature. After washing with 0.2% NP-40 in PBS three times and water once, the immunocomplexes were visualized by autoradiography.

Immunofluorescence

For RFCs, coverslips were coated with either human vitronectin (3 or 30 μg/ml) or mouse laminin (10 μg/ml), as described above. For NIH-3T3 cells, coverslips were coated with either human vitronectin (3 μg/ml) or rat laminin (30 μg/ml). Cells were resuspended in DMEM containing 0.5% serum, seeded on the coated coverslips and incubated at 37°C for 18 hours. The cells were stimulated with 30 ng/ml PDGF-BB for 10 minutes or left quiescent, fixed with 3% paraformaldehyde for room temperature for 10 minutes. Cells were permeabilized with 0.1% Triton X-100 in PBS at room temperature for 5 minutes and subsequently blocked with 5% BSA in PBS for 2 hours at room temperature. Primary antibodies were diluted in PBS containing 0.1% BSA and incubated with the fixed cells at room temperature for 2 hours. The rabbit antisera against the αvβ3 cytoplasmic domain (provided by Erkki Ruoslahti) was used at a dilution of 1:250 for RFCs and 1:100 for NIH-3T3 cells. The coverslips were then incubated at room temperature for 30 minutes with horse serum (Gemini Bio-Products, Inc., Calabasas, CA), diluted 1:50 in PBS. FITC-conjugated goat anti-rabbit secondary antibody (Jackson Labs, West Grove, PA) was diluted 1:250 for RFCs and 1:200 for NIH-3T3 cells in PBS containing 0.1% BSA and incubated at room temperature for 45 minutes in the dark. Each coverslip was mounted on a drop of Slow Fade Antifade Kit from Molecular Probes (Eugene, OR). The results were analyzed using a Bio-Rad MRC 600 inverted confocal microscope. For time course experiments, RFCs were plated on vitronectin and stimulated with PDGF-BB, as described above, for 10 minutes, 1, 2, 3 or 4 hours. The cells were then fixed and stained as described above.

Immunoprecipitation

Serum starved cells were stimulated with 3 ng/ml PDGF-BB at 37°C for 10 minutes. Cells were then washed twice with ice-cold PBS, and lysed in the 1% Triton X-100 lysis buffer described in the affinity chromatography section. Lysates were precleared with nonimmune rabbit serum and Protein A-Sepharose (Sigma) at 4°C. Lysates were then incubated overnight at 4°C with rabbit antisera raised against: the human placental vitronectin receptor, αvβ3 (providing by Elisabetta Dejana, Mario Negri Institute, Milan, Italy), the PDGFRα (antiserum (1) from Ron Seifert (University of Washington, Seattle, WA) or antiserum (2) from Thomas O. Daniel (Vanderbilt University Medical Center, Nashville, TN); both antisera crossreact with human, mouse and rat PDGFRβ), the human αvβ3 integrin (Life Technologies, Inc.) (this antibody immunoprecipitates murine αβ3 from NIH-3T3 cells; Bartfeld et al., 1993), the cytoplasmic domain of the human β3 integrin subunit, or preimmune serum. After incubation with Protein A-Sepharose, immunoprecipitates were washed three times with lysis buffer containing 0.35 M NaCl and two more times using lysis buffer. The immunocomplexes were then resuspended in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 M dithiothreitol (Bio-Rad Laboratories), 0.1% Bromophenol Blue (Bio-Rad Laboratories)). For immunoprecipitations, immunocomplexes were resuspended in 50 mM Tris-HCl, pH 7.5, 2% SDS and heated at 95°C for 5 minutes. Supernatants were diluted 10-fold using lysis buffer and incubated with either preimmune, or anti-PDGFRβ(2), or antisera overnight at 4°C. After incubation with Protein A-Sepharose, the immunoprecipitates were washed and resuspended, as indicated above. Immunoprecipitates were separated by SDS-PAGE (7.5%) and transferred either to Immobilon P transfer membranes (Millipore, Burlington, MA) for PY20 immunoblotting or to nitrocellulose membranes for anti-β3 immunoblotting. Immunoblotting was performed using either 1 μg/ml anti-phosphotyrosine monoclonal antibody, PY20 (ICN) as previously described (Lin et al., 1995) or rabbit antisera against the cytoplasmic domain of the human β3 integrin subunit as described above with the following modifications: a peroxidase-conjugated goat affinity purified antibody to rabbit IgG (Cappel, Durham, NC) was used as secondary antibody and immunocomplexes were detected using ECL (Amersham).

RESULTS

PDGF-BB stimulates cell migration on vitronectin

In order to examine a potential synergistic effect of the engagement of αvβ3 and the PDGFRβ in the regulation of microvascular cell functions, we determined the effect of PDGF-BB stimulation on RFC migration on vitronectin, an αvβ3 ligand. Laminin, a ligand for the β1 integrins, was selected as a control substrate. The concentration of vitronectin and laminin used for coating was selected to have comparable levels of cell attachment to the migration inserts (data not shown). Fig. 1A shows that PDGF-BB specifically increased migration of RFCs plated on human vitronectin (3.7-fold compared to resting cells). This effect was further enhanced in the presence of a PDGF-BB concentration gradient (9.2-fold). In contrast, the cells did not migrate on laminin in the absence of a PDGF-BB concentration gradient. In the presence of a PDGF-BB concentration gradient, background levels of migration were observed on laminin or BSA (2.4 or 2.0-fold, respectively). In order to confirm that the observed effect on cell migration was mediated by αvβ3 and to exclude the role of non-RGD binding receptors for vitronectin, such as the urokinase receptor (Wei et al., 1994), we tested the ability of a RGD containing peptide to inhibit the PDGF-BB induced migration of RFCs on vitronectin. Fig. 1B shows that the RGD containing peptide completely blocked the PDGF-BB stimulated migration of RFCs on vitronectin, while a RGE containing control peptide had no effect. Due to the ability of RGD to inhibit αvβ3, αvβ3 and αvβ3 binding to vitronectin (Felding-Habermann and Chersch, 1993), the specific role of αvβ3 in PDGF-BB stimulated cell migration was investigated using a purified polyclonal antibody against αvβ3. Fig. 1C shows that the antibody against αvβ3 inhibits PDGF-BB stimulated migration on vitronectin, while control IgGs against β1c had no effect. The migratory effect of PDGF-BB was also examined in NIH-3T3 cells. Fig. 1D illustrates that PDGF-BB specifically increased a 7.3-fold increase in migration of NIH-3T3 cells on vitronectin and no increase on laminin. The PDGF-BB induced migration of NIH-3T3 cells on vitronectin could be blocked by herbimycin A, a tyrosine kinase inhibitor (data not shown). These results show that the increase in migration of RFCs and NIH-3T3 cells in response to PDGF-BB stimulation is dependent upon αvβ3 engagement. We investigated whether the increase in RFC and NIH-3T3 cell migration on vitronectin in response to PDGF-BB was due
to an increase in adhesion of the cells to vitronectin. Fig. 2A shows that RFC adhesion was not altered by the presence of PDGF-BB. The same experiment was performed using NIH-3T3 cells. Fig. 2B illustrates that adhesion of NIH-3T3 cells to vitronectin was not altered by PDGF-BB stimulation. Thus, the specific increase in migration of RFCs and NIH-3T3 cells on vitronectin in response to PDGF-BB stimulation is not due to an increase in cell adhesion.

To determine whether the observed increase in migration was associated with an increase in proliferation, we examined the effect of PDGF-BB on cell proliferation on vitronectin or laminin. The concentration of vitronectin or laminin used for coating in the proliferation assay was shown to generate comparable levels of cell attachment prior to the experiment (data not shown). In Fig. 3, proliferation assays of cells plated on either vitronectin or laminin were performed in the presence or absence of PDGF-BB. RFCs proliferated to a similar extent on vitronectin or laminin (Fig. 3A). In these experimental conditions, RFC adhesion to vitronectin or laminin was comparable in the presence or absence of PDGF-BB (Fig. 3B). Proliferation assays were also performed using NIH-3T3 cells, as described above. Fig. 3C shows that the increase in proliferation in response to PDGF-BB stimulation was comparable on vitronectin or laminin. Therefore, the increase in migration of RFCs and NIH-3T3 cells on vitronectin in response to PDGF-BB stimulation did not correlate with an increase in cell proliferation.

**PDGF-BB does not alter αvβ3 binding to RGD**

In order to examine whether PDGF-BB stimulation altered the ability of αvβ3 to bind RGD, that represents the vitronectin binding site for αvβ3, we purified αvβ3 from PDGF-BB stimulated or resting NIH-3T3 cell lysates, using RGD-Sepharose affinity chromatography. Cells were then harvested, lysed and αvβ3 was purified using a RGD-Sepharose column, as described in Materials and Methods. Fig. 4 is an immunoblot of the eluted fractions with an antibody raised against the human β3 integrin subunit. Comparable amounts of αvβ3 were purified on the RGD-Sepharose column from either PDGF-BB stimulated or resting cells (Fig. 4). Thus, PDGF-BB stimulation of NIH-3T3 cells did not alter the ability of αvβ3 to bind RGD.
Effect of PDGF-BB on $\alpha_v$ localization

To determine whether PDGF-BB stimulation would affect $\alpha_v$ localization in focal contacts, we analyzed the distribution of $\alpha_v$ via immunofluorescence in both cell types. Using a rabbit antiserum against the $\alpha_v$ cytoplasmic domain, we found that $\alpha_v$ was localized to focal contacts in RFCs in the presence (Fig. 5A,C) or the absence of PDGF-BB stimulation (Fig. 5B,D). The RFCs were attached to two different concentrations of vitronectin (3 or 30 $\mu$g/ml in 5A,B or 5C,D, respectively) since these concentrations were used in the previously described migration or proliferation assays. Additionally, $\alpha_v$ was localized to focal contacts in both the presence or absence of PDGF-BB when RFCs were plated on laminin (data not shown). The same expression pattern of $\alpha_v$ in focal contacts on vitronectin was observed when RFCs were stimulated with PDGF-BB for 1, 2, 3, or 4 hours (data not shown).

In striking contrast to the RFCs, NIH-3T3 cells plated on vitronectin showed a diffuse staining of $\alpha_v$ after PDGF-BB stimulation (Fig. 6A). Prior to PDGF-BB stimulation, $\alpha_v$ was found in focal contacts when NIH-3T3 cells were plated on vitronectin (Fig. 6B). The PDGF-BB induced loss of $\alpha_v$ localization in focal contacts is specific for vitronectin since there was no effect observed when NIH-3T3 cells were plated on laminin (Fig. 6C,D), although fewer focal contacts stained positively for $\alpha_v$ on this substrate in both the presence or absence of PDGF-BB. This result is not surprising since unlike vitronectin, laminin is not a ligand for $\alpha_v$ containing integrins. An antibody to phosphotyrosine showed the same results obtained using the antibody against the $\alpha_v$ cytoplasmic domain (data not shown). These data show a cell-type dependent loss of $\alpha_v$ in focal contacts in response to PDGF-BB stimulation.

The PDGFR$\beta$ is associated with $\alpha_v\beta_3$

To examine the mechanism by which both RFCs and NIH-3T3 cells preferentially migrate on vitronectin in response to PDGF-BB, we examined the possibility that $\alpha_v\beta_3$ and the PDGFR$\beta$ might be associated. PDGF-BB stimulated tyrosine phosphorylation of the PDGFR$\beta$ in RFCs and NIH-3T3 cells (Fig. 7B, lane 1 and 7D,E, lanes 3). Immunocomplexes containing $\alpha_v\beta_3$ or $\alpha_v\beta_1$ were reprecipitated using antibodies against the PDGFR$\beta$, and proteins were detected by immunoblotting with an antibody to phosphotyrosine, as described in Materials and Methods. Fig. 7A (lane 1) shows that the PDGFR$\beta$ was present in the $\alpha_v\beta_3$ immunocomplexes in RFCs. Fig. 7C,D,E show that $\alpha_v\beta_3$ and the PDGFR$\beta$ are also associated in PDGF-BB stimulated NIH-3T3 cells. In contrast, Fig. 7F, shows that the PDGFR$\beta$ is not coprecipitated with $\alpha_v\beta_1$. The results were comparable using two different antibodies generated against the human vitronectin receptor ($\alpha_v\beta_3$) or the platelet $\alpha_v\beta_3$ (Fig. 7C,D). In lanes 1 of Fig. 7C,D, the PDGFR$\beta$ and $\alpha_v\beta_3$ were coprecipitated using both antibodies. We also tested two different rabbit antisera against the PDGFR$\beta$ in the reprecipitation and showed that they specifically reprecipitated the PDGFR$\beta$ from $\alpha_v\beta_3$ immunocomplexes (lanes 1 of Fig. 7D,E). In a previous study (Bartfeld et al., 1993), we did not detect PDGFR$\beta$ in the $\alpha_v\beta_3$ immunocomplexes; and this was likely due to the less sensitive approach that was used. Coimmunoprecipitation was also shown by immunoblotting of PDGFR$\beta$ immunoprecipitates using the antiserum against the cytoplasmic domain of the $\beta_3$ integrin subunit (Fig. 8).

In summary, the $\alpha_v\beta_3$ integrin and the PDGFR$\beta$ are specifically associated in PDGF-BB stimulated RFCs and NIH-3T3 cells.

DISCUSSION

We have shown that $\alpha_v\beta_3$ and the PDGFR$\beta$ act in a synergistic manner in modulating migration of RFCs and NIH-3T3 cells on vitronectin. The PDGF-BB increased cell migration on vitronectin occurred specifically in response to $\alpha_v\beta_3$ engagement, since the same effect was not observed upon $\beta_1$ integrin engagement by laminin. PDGF-BB did not change the
cells’ ability to adhere to vitronectin, nor did it stimulate a further increase in proliferation on vitronectin versus laminin, nor did it change the ability of \( \alpha_5 \beta_1 \) to bind RGD. We also demonstrate that \( \alpha_5 \beta_3 \), but not \( \alpha_5 \beta_1 \), coprecipitates with the PDGFR\( \beta \) in PDGF-BB stimulated cells, indicating that their association might play a crucial role in the regulation of cell migration on a specific matrix.

The synergistic activity of \( \alpha_5 \beta_3 \) and the PDGFR\( \beta \) does not appear to be cell type specific, since it occurs in both microvascular endothelial cells and fibroblasts. Previous studies have underscored the role of \( \alpha_5 \beta_3 \) in smooth muscle cell migration on vitronectin (Clyman et al., 1992). It would be of interest to determine if the same synergy occurred in smooth muscle cells, since these are the first cells to respond to PDGF stimulation in a pathological or physiological vascular response. Schneller et al. (1997) have also very recently shown an association of \( \alpha_5 \beta_3 \) and the PDGFR\( \beta \) in NIH-3T3 cells and described a synergistic activity of PDGF-BB and \( \alpha_5 \beta_3 \) on foreskin fibroblast migration, further suggesting that this pathway is common to a wider range of cell types. However, in this study, in contrast to our observations, the effect on cell migration correlated with an
increased proliferation on vitronectin, indicating that cell type specificity (NIH-3T3 or RFCs versus foreskin fibroblasts) may differentially modulate the cell proliferation response to the combined activities of integrins and growth factors.

Ours is the first report showing a synergistic effect between \( \alpha_v\beta_3 \) and PDGF-BB in microvascular endothelial cells. The presence of the PDGFR\( \beta \) has been extensively documented in microvascular endothelial cells (Bar et al., 1989; Beitz et al., 1991; Hermansson et al., 1988; Marx et al., 1994; Plate et al., 1992; Smits et al., 1989; Streten et al., 1989). Because \( \alpha_v\beta_3 \) and PDGF-BB have been shown to be mediators of angiogenesis (Brooks et al., 1994a; Gamble et al., 1993; Risau et al., 1992), their physical association may be important in a previously uncharacterized pathway that leads to neovessel formation. Furthermore, since angiogenesis supports tumor growth (Brooks et al., 1994b, 1995), the association of \( \alpha_v\beta_3 \) and the PDGFR\( \beta \) deserves further study to investigate a potential role for these receptors in cancer. Our in vitro findings are also likely to be relevant to pathological conditions of the vascular system. Because vitronectin is a plasma protein (Preissner, 1991) and PDGF is released by both the endothelium (Fox and DiCorleto, 1991) and platelets upon agonist stimulation (Claesson-Welsh, 1996), it is likely that both ligands are in close proximity to the endothelium surface and might, therefore, interact with the injured vessel wall.

A role for the \( \alpha_v\beta_3 \) integrin in mediating microvascular endothelial cell migration on osteopontin in response to VEGF has been shown (Senger et al., 1996); however, in our experimental system, an inconsistent effect of VEGF on the migration of microvascular cells on vitronectin or laminin coated substrates was observed (A. S. Woodard, J. A. Madri and L. R. Languino, unpublished observations).

The specificity of the association between \( \alpha_v\beta_3 \) and the PDGFR\( \beta \) was illustrated by performing immunoprecipitations using two different rabbit polyclonal antisera to either \( \alpha_v\beta_3 \) integrin complexes or to the PDGFR\( \beta \). The failure of \( \alpha_v\beta_3 \) to coprecipitate with the PDGFR\( \beta \) indicates that \( \beta_1 \) integrins do not play a role in the described pathway. Thus, the role of the vitronectin receptor ligands is unique among the large number of

**Fig. 5.** \( \alpha_v \) localization in RFCs spread on vitronectin: effect of PDGF-BB. RFCs were plated in DMEM with 0.5% serum on coverslips which had been previously coated with human vitronectin at 3 \( \mu \)g/ml (A,B) or 30 \( \mu \)g/ml (C,D) and allowed to attach for 18 hours. (A,C) The cells were stimulated with 30 ng/ml PDGF-BB for 10 minutes; (B,D) the cells were left quiescent. Cells were fixed and stained with rabbit antiserum against the \( \alpha_v \) cytoplasmic domain as described in Materials and Methods. The results were then analyzed using a Bio-Rad MRC 600 inverted confocal microscope. No differences were observed in PDGF-BB stimulated versus resting RFCs.

**Fig. 6.** \( \alpha_v \) localization in NIH-3T3 cells spread on vitronectin: effect of PDGF-BB. NIH-3T3 cells were plated in DMEM containing 0.5% serum on coverslips which had been previously coated with human vitronectin at 3 \( \mu \)g/ml (A,B) or rat laminin at 30 \( \mu \)g/ml (C,D) and allowed to attach for 18 hours. (A,C) The cells were stimulated with 30 ng/ml PDGF-BB for 10 minutes; (B,D) the cells were left quiescent. Cells were fixed and stained with rabbit antiserum against the \( \alpha_v \) cytoplasmic domain as described in Materials and Methods. The results were then analyzed using a Bio-Rad MRC 600 inverted confocal microscope. Loss of \( \alpha_v \) from focal contacts in PDGF-BB stimulated NIH-3T3 cells was observed.
PDGFR immunoblotted using PY20 to detect the phosphorylated in SDS sample buffer, separated by SDS-PAGE and buffer. The immunoprecipitates were either resuspended in Sepharose, immunoprecipitates were washed with lysis buffer. The immunoprecipitates were resuspended in lysis buffer. After incubation with Protein A-Sepharose, immunoprecipitates were washed with lysis buffer. The immunoprecipitates were either resuspended in SDS sample buffer, separated by SDS-PAGE and immunoblotted using PY20 to detect the phosphorylated PDGFRβ or, in the case of reprecipitation, resuspended in 50 mM Tris-HCl pH 7.5, 2% SDS and heated at 95°C for 5 minutes. Supernatants were diluted with lysis buffer and reprecipitated with the indicated antibodies, washed, resuspended in SDS sample buffer, separated by SDS-PAGE and immunoblotted using PY20. (Note: tyrosine phosphorylation of αβ3 was never observed in our experimental system).

Other extracellular matrix proteins, that bind β1. However, it should be noted that an example of β1 integrin clustering with growth factor receptors (PDGFRβ and α, epidermal growth factor (EGF) receptor and bFGF receptor; Miyamoto et al., 1996), has recently been shown, although it occurred transiently in response to β1 integrin engagement by fibronectin or by a functional antibody to β1, thus suggesting that β1 might be involved in, as yet uninvestigated, growth factor stimulated pathways.

It remains to be determined whether the association of αβ3 and the PDGFRβ is a direct interaction or if other proteins are involved. In a previous report (Bartfeld et al., 1993), we described a 190 kDa protein that is associated with αβ3 and tyrosine-phosphorylated in response to PDGF-BB. Preliminary results based on internal amino acid sequence show that the 190kDa is distinct (L. R. Languino, unpublished observations) from the PDGFRβ; however, this study is currently in progress and it requires further investigations.

Our study shows that the localization of the αv integrin in RFCs and NIH-3T3 cells is differentially affected by PDGF-BB. In NIH-3T3 cells plated on vitronectin, there was a complete loss of focal contact staining of the αv integrin after PDGF-BB stimulation. The same loss of focal contact staining was observed when immunofluorescence was performed using a monoclonal antibody against phosphorysine (data not shown). The redistribution of αv was specific for NIH-3T3 cells plated on vitronectin, since the same effect was not observed when cells were plated on laminin. Surprisingly, there was no redistribution of αv in RFCs plated on vitronectin and subsequently stimulated with PDGF-BB, indicating that the αv loss from focal contacts is not a prerequisite for the observed changes in cell migration.

At this time, we do not know the identity of the downstream intracellular signaling molecules that are synergistically activated by engagement of both αβ3 and the PDGFRβ. Focal adhesion kinase (FAK) is an intracellular target for both αβ3 and the PDGFRβ (Lewis et al., 1996; Rankin and Rozengurt, 1994). FAK is a nonreceptor tyrosine kinase localized in focal contacts that becomes tyrosine phosphorylated and immediately activated in response to integrin engagement (Otey, 1996; Tahiliani et al., 1997). FAK is also involved in mediating signal transduction from the extracellular environment during cell migration (Cary et al., 1996; Gilmore and Romer, 1996; IIIc et al., 1995; Sankar et al., 1995). Therefore, FAK activation, potentiated by the engagement of both αβ3 and the PDGFRβ, is likely to be one of the earliest events in the cascade that ultimately modulates cytoskeletal reorganization and cell migration. Further investigations in this area will provide new insights into the mechanisms that regulate cell migration in physiological and pathological conditions.

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Modulation of cell spreading and migration by pp125FAK phosphorylation. Am. J. Pathol. 147, 601-608.


