Platelet-derived Growth Factor Inhibits Smooth Muscle Cell Adhesion to Fibronectin by ERK-dependent and ERK-independent Pathways*

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The adhesion of cells to the extracellular matrix plays a major role in cell migration. Pretreatment with platelet-derived growth factor (PDGF) inhibited the adhesion of smooth muscle cells to fibronectin by 80%. This inhibition decreased as concentrations of fibronectin increased. In the presence of 200 \( \mu \)G RDGS peptide, only 45% of PDGF-treated cells adhered to fibronectin compared with 80% of control cells. This indicates that a decrease in integrin avidity was induced by PDGF. Cell adhesion was partially restored when the activation of the extracellular signal-regulated kinase (ERK) was inhibited with PD98059. The remaining inhibition of adhesion (50%) was independent of the fibronectin concentration, suggesting that the ERK pathway is involved in the decrease in integrin avidity. This was confirmed by depleting ERK protein levels by treatment with ERK antisense oligonucleotide. The adhesion of ERK control oligonucleotide-treated cells decreased by 41% when the concentration of GRGDS peptide was increased from 50 to 200 \( \mu \)G but only decreased by 11% in ERK antisense oligonucleotide-treated cells. Treatment with PDGF also delayed focal complex assembly and inhibited stress fiber formation. Consistent with a delay in tyrosine phosphorylation of paxillin, PDGF treatment caused a lag in focal complex formation, although this was not associated with any change in Src family tyrosine kinase activity. Our results indicate that PDGF inhibits smooth muscle cells adhesion by two pathways. The first involves an ERK-dependent decrease in integrin avidity; the second involves the ERK-independent inhibition of focal complex assembly.

A key event in the development of atherosclerotic lesions is the migration of smooth muscle cells (SMC)1 from the media to the intima where they proliferate and produce excess connective tissue components (1–3). Cell migration involves numerous cellular processes that are spatially and temporally coordinated and occur in four steps, the formation and adhesion of protrusive structures at the leading edge, the translocation of the cell body and nucleus, and rear detachment (4, 5). Cell adhesion appears to be a major component of cell migration. Moreover, cell migration is controlled by the strength of the interactions between the cell and the substrate. When the cells are highly adhesive they spread but do not move; however, the connections between the extracellular matrix and the cell surface must be firm enough to pull the cell forward. Maximal cell migration occurs at an intermediate level of cell-substratum adhesiveness (6, 7), which depends on integrin-ligand interactions and cytoskeletal organization (8).

Intracellular signaling, called “inside-out signaling,” controls the interaction between integrins and ligands. This cell type-specific process (9) increases receptor affinity, which is brought about by a conformational change and modulation of lateral diffusion and/or receptor clustering (9, 10). Integrin affinity is defined by a dissociation constant and characterizes the interaction between integrins and soluble ligands. However, it cannot be used to characterize multivalent ligand binding. Integrin avidity characterizes this interaction and involves both affinity and receptor clustering. Affinity modulation is probably the predominant regulator of cell adhesion rather than receptor clustering (11). Suppression of integrin activation via a Ras/Raf-signaling pathway (12) might modulate cell-substratum adhesiveness in growth factor-induced cell migration, but the consequences on cell adhesion are unknown.

Contacts between the cell and the extracellular matrix are mediated by adhesive structures called “focal adhesions,” which are found at the end of bundles of actin filaments. Focal adhesions are composed of multiple cytoskeletal and membrane components linked to large aggregates of integrins (13). These structures are present in stationary cells but not in motile cells in which protrusive structures form small attachment sites called “focal complexes” (14). The disassembly of focal adhesions is required for cell movement (15) and is induced by growth factors that promote cell migration (16). However, the mechanisms by which they mediate their effects are not clearly established. Directed migration toward platelet-derived growth factor (PDGF) has been proposed to involve Ras activation and the binding of both phospholipase C\(\gamma\) and phosphoinositide 3-kinase to the PDGF receptor (17, 18).

Several studies (19–21) using animal injury models indicate that PDGF is an important SMC chemoattractant but a poor mitogen and that it plays a key role in the migration of medial SMC (3, 4). To assess the involvement of adhesion modulation in PDGF-induced cell mobility, we investigated the effects of PDGF on SMC adhesion to fibronectin. We found that PDGF inhibited SMC adhesion by two different pathways, 1) an ERK-dependent pathway, which decreases integrin avidity and adhesion when fibronectin-coating concentrations are low, and 2) an ERK-independent pathway, which decreases adhesion at all fibronectin-coating concentrations and is associated with delayed formation of focal complexes.

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The abbreviations used are: SMC, smooth muscle cells; ERK, extracellular signal-regulated kinase; PDGF, platelet-derived growth factor; BSA, bovine serum albumin.

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FIG. 1. Adhesion of SMC to fibronectin: effects of pretreatment with PDGF. A, SMC were plated in wells coated with different concentrations of fibronectin. Cell adhesion was measured by the endogenous acid phosphatase assay as described under “Experimental Procedures.” The percentage of cell adhesion at each fibronectin concentration is expressed as a percentage of maximal adhesion obtained at 5 μg/ml fibronectin. B, suspended SMC were treated with various concentrations of PDGF for 1 h before plating on 0.5 μg/ml (open circles) or 5 μg/ml (closed circles) of fibronectin. Data are expressed as the percentage of cell adhesion in the absence of PDGF. Data in both graphs represent the mean ± S.E. of three independent experiments, carried out in triplicate.

EXPERIMENTAL PROCEDURES

Reagents—Culture media and reagents were obtained from Life Technologies. Trypsin and soybean trypsin inhibitor were obtained from Worthington Biochemical Corporation (Lakewood, NJ). Fibronectin and enolase were purchased from Roche Molecular Biochemicals. Recombinant human PDGF-BB was obtained from R&D Systems (Minneapolis, MN). PD98059 and PP1 were purchased from Biomol (Plymouth Meeting, PA). GRGDS or control GRADSP peptides were purchased from Calbiochem-Novabiochem. Oligodeoxynucleotides were synthesized by Sigma. SuperFect Transfection Reagent was purchased from Qiagen (Valencia, CA). Protein concentrations were determined by use of a DC protein assay kit from Bio-Rad. Anti-paxillin, anti-phospho-tyrosine (PY20), and anti-ERK1 mouse monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antibody directed against the conserved C-terminal sequence 509–533 of Src family tyrosine kinases and anti-ERK2 rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa 488-labeled phallolidin was purchased from Molecular Probes (Eugene, OR). Protein A-Sepharose was obtained from Amersham Pharmacia Biotech. 1×23ATP (3000 Ci/mmol) was bought from PerkinElmer Life Sciences.

Cell Culture—Primary cultures of SMC were established from media explants of pig aortas and were subsequently cultured up to seven passages in minimum essential medium with Earle’s salts containing 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum (22). Cultures were rendered quiescent by incubation in serum-free medium containing 0.2% (w/v) BSA for 96 h. The cells were placed into fresh medium 24 h before the experiments.

TREATMENT OF CELLS WITH ANTISENSE ERK Oligonucleotides—Mitogen-activated protein kinases (ERK1 and ERK2) were depleted by treating SMC with antisense phosphorothioate oligoexonucleotides directed against the ERK1 and ERK2 translation initiation site. SMC were transfected 24 h after plating at 70–80% confluence with 0.4 μM ERK antisense (5′-AGC CGC CCG CGC CGC CAT-3′) or control (5′-CCG CGC CTC GCA GCA CGC CCC-3′) oligonucleotides using the transfection reagent SuperFect at a ratio of 5 μL of liposome per μg of oligonucleotide. SMC were incubated with the transfection complexes for 4 h in the presence of 10% serum, which was then replaced with fresh medium, and finally, cells were made quiescent by serum starvation for 48 h.

PREPARATION OF CELLS FOR ADHESION EXPERIMENTS—Twenty-four well plates were coated with fibronectin overnight at 4 °C. Non-specific adherence was blocked by incubation with 0.5% BSA in phosphate-buffered saline for 1 h at 37 °C. Quiescent SMC were harvested by limited trypsinization with 0.05% trypsin, 0.02% EDTA. Cells were washed in minimum essential medium with Earle’s salts (MEM) containing 0.2% BSA and 100 μg/ml soybean trypsin inhibitor and then resuspended in MEM containing 0.2% BSA. Cells were maintained in suspension in BSA-coated plates for 30 min before the addition of PDGF, incubated for 1 h, and plated out. The GRGDS peptide was added 30 min before plating. When appropriate, PD98059 was added to the suspended cells. The cells were incubated for 1 h before the addition of PDGF and then incubated for a further hour. Suspensions of control cells were incubated in PDG1 inhibitor for 30 min before plating.

Adhesion Assay—Suspended SMC were transferred to fibronectin-coated wells at a density of 5 × 10^4 cells/well. After 30 min at 37 °C, adherent cells were quantified by the acid phosphatase assay (23). The absorbance was read at 405 nm, and nonspecific adhesion was determined in wells coated with 0.5% BSA.

Spreading Assay—Suspended SMC were plated on fibronectin-coated wells (5 μg/ml). After 60 min at 37 °C, adherent cells were fixed with 70% ethanol and stained with 0.2% (w/v) crystal violet in 2% (v/v) ethanol/water for 30 min at room temperature and then washed with distilled water. Spread cells were not phase-bright, whereas unspread cells were phase-bright when viewed under a microscope. At least 1,000 cells were counted for each experimental condition.

Immunofluorescence Microscopy—Suspended SMC were allowed to spread on fibronectin-coated coverslips (5 μg/ml) at 37 °C for different times. Adherent cells were fixed in 4% paraformaldehyde for 15 min and permeabilized for 30 min in 1% Triton X-100 in permeabilization buffer (300 mM sucrose, 20 mM HEPES, 50 mM NaCl, 3 mM MgCl2, pH 7.0). Cells were then blocked in phosphate-buffered saline containing 5% BSA for 1 h. Filamentous actin was visualized by use of Alexa 488-labeled phallolidin. Focal adhesions were stained with an anti-paxillin antibody and a Texas red-conjugated secondary antibody.

Immunoprecipitation—Suspended SMC were plated out on fibronectin-coated culture dishes (5 μg/ml) and incubated at 37 °C for different times. Adherent cells were harvested at the time of immunoprecipitation using lysis buffer containing 50 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, 1% Tween 20, 0.5% aprotinin, 10 μg/ml leupeptin. The lysates were centrifuged at 15,000 × g for 15 min at 4 °C, and the protein concentration was determined. Monoclonal antibodies directed against paxillin or polyclonal antibodies directed against the Src family tyrosine kinases, SRC2, were added to 200 μg of cell lysate and incubated for 2 h at 4 °C. Immune complexes were collected on protein A-Sepharose either after the addition of rabbit anti-mouse IgG or not. They were subsequently washed three times in lysis buffer without deoxycholate or SDS at 4 °C and once in a buffer containing 50 mM HEPES, 150 mM NaCl, and 0.1% Triton X-100, pH 7.4. Samples were separated on 6% SDS-polyacrylamide gels or were subjected to an in vitro kinase assay.

Immunoblotting—Tyrosine phosphorylation of paxillin was analyzed by immunoblotting with the PY20 monoclonal phosphotyrosine antibody. Membranes were reprobed with anti-paxillin antibody to ensure that equal amounts of protein were immunoprecipitated. Total cell lysates from suspended cells were also analyzed by immunoblotting with the anti-ERK2 polyclonal antibody. Bound primary antibodies were visualized by enhanced chemiluminescence detection with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies.

Kinase Assay—Src kinase activity was assessed by the enolase assay. Src immunoprecipitates were washed as described above and then twice with kinase buffer, pH 7.0 (100 mM HEPES, 10 mM MgCl2, 10 mM MnCl2). Immunoprecipitates were resuspended in kinase buffer con-
the concentration of the GRGDS peptide increased (0 μM). It was suspected that cell adhesion was mediated by the endogenous acid phosphatase assay. Data are expressed as the percentage of cell adhesion in the absence of the GRGDS peptide (A) and in the absence of PDGF (B). Data represent the mean ± S.E. of four independent experiments, carried out in triplicate.

RESULTS

PDGF Inhibits Adhesion of SMC to Fibronectin—We first examined the effects of fibronectin concentration on the adhesion of quiescent SMC. Ninety percent of suspended SMC rapidly adhered to fibronectin (5 μg/ml). Maximal binding occurred by 30 min. When the fibronectin-coating concentration was 0.5 μg/ml, cell attachment was 34 ± 2% of this maximal binding (Fig. 1A). To examine the receptors involved in adhesion we used the GRGDS peptide, which prevents integrin-mediated cell adhesion to fibronectin. The addition of 2 mM GRGDS peptide inhibited 80% of SMC adhesion to 5 μg/ml fibronectin, whereas 2 mM inactive peptide GRADSP had no effect (data not shown). This indicates that integrins mediate this effect of PDGF inhibition of SMC to fibronectin.

Pretreatment with PDGF decreased SMC adhesion to fibronectin. The maximal effect occurred at low PDGF concentrations (0.5 ng/ml) and required a 30-min pretreatment period (data not shown). PDGF (2 ng/ml) inhibited the adhesion of SMC to 5 μg/ml fibronectin by 51 ± 9% (p < 0.002) and to 0.5 μg/ml fibronectin by 84 ± 4% (Fig. 1B). This shows that the effect of PDGF depends on the fibronectin concentration. These results show that pretreatment with PDGF inhibits SMC adhesion and suggests that PDGF modulates the integrin avidity of SMC.

PDGF Decreases Avidity of Integrins—To determine the effects of PDGF on integrin avidity, we studied the inhibition of cell adhesion to fibronectin by the GRGDS peptide (0 to 200 μM). In the absence of PDGF, cell adhesion was slightly inhibited (19 ± 3%; p < 0.001) by 200 μM GRGDS peptide (Fig. 2A). In contrast, in the presence of PDGF, cell adhesion was inhibited by 40 ± 3% by 50 μM and 55 ± 4% by 200 μM of GRGDS peptide (p < 0.001 compared with the control). PDGF-treated cells were more sensitive to the GRGDS peptide. PDGF induced a parallel increase in the inhibition of cell adhesion as the concentration of the GRGDS peptide increased (0–200 μM). However, no significant variations in cell adhesion was observed in the presence of higher concentrations of GRGDS peptide (200–1000 μM), suggesting that PDGF does not modulate integrins in the highest activation states (Fig. 2B). These results indicate that PDGF regulates integrin-mediated SMC adhesion by decreasing receptor avidity.

The ERK Pathway Mediates a Decrease in Integrin Avidity—It has been reported that the Ras-linked mitogen-activated protein kinase pathway mediates the suppression of integrin activation (12). Therefore, we assessed the involvement of the ERK pathway in the inhibition of SMC adhesion by PDGF. Cells were maintained in suspension for 1 h in the presence of 0–20 μM PD98059, an inhibitor of MEK1/2 activation and were subsequently stimulated with PDGF (2 ng/ml) for 10 min. The phosphorylation state, which correlates with a reduction in the electrophoretic mobility of ERK2, was investigated by Western blotting using an antibody that recognizes phosphorylated and non-phosphorylated ERK2. Suspended cells retained the ability to activate ERK2 in response to PDGF. PD98059 inhibited the phosphorylation of ERK2 in a dose-dependent manner, with maximal inhibition at 20 μM (Fig. 3A). In parallel, the effect of PD98059 on the inhibition of cell adhesion induced by PDGF was tested (Fig. 3B). In the absence of the inhibitor, cell adhesion to 0.5 μg/ml fibronectin was inhibited by 76 ± 3%. This decreased to 49 ± 4% (p < 0.001) when PD98059 (20 μM) was added. The decrease was only slight in the presence of...
Moreover, in the presence of PD98059, inhibition of SMC shown.

Cells (lane Sc, treated with control oligonucleotides; lane As, treated with antisense oligonucleotides. Blots were reprobed with an anti-α-actin antibody to ensure equal loading. The ERK levels are expressed as a percentage of ERK relative to α-actin levels in control cells (B, C, and D). SMC pretreated with PDGF (2 ng/ml) were incubated for 30 min in the presence of increasing concentrations (0, 50, or 200 μM) of the GRGDS peptide and plated in wells coated with fibronectin (5 μg/ml). Cell adhesion was measured by the endogenous acid phosphatase assay. B, data are expressed as a percentage of cell adhesion in the absence of the GRGDS peptide. C, data are expressed as a percentage of cell adhesion in the presence of 50 μM GRGDS peptide. All data represent the mean ± S.E. of three independent experiments, carried out in triplicate. D, data are expressed as a percentage of cell adhesion in the absence of PDGF. Representative results from three independent experiments are shown.

5 μg/ml fibronectin (from 56 ± 2% to 47 ± 2%, p < 0.05). Moreover, in the presence of PD98059, inhibition of SMC adhesion to 0.5 μg/ml and 5 μg/ml fibronectin did not differ significantly. Our results indicate that the inhibition of SMC adhesion induced by PDGF is independent of the fibronectin concentration when ERK is not activated.

To establish further the involvement of the ERK signaling pathway in the decreased integrin avidity, we down-regulated the amount of ERK protein by use of an oligonucleotide that targets the ERK1 and ERK2 translation initiation sites, which are identical. Levels of the ERK proteins were decreased by 60–70% in cells treated with an ERK antisense oligonucleotide. The controls consisted of cells treated with transfectant reagent alone or with scrambled control oligonucleotides (Fig. 4A). In parallel, we investigated the effect of the GRGDS peptide on the adhesion of PDGF-treated cells. Treatment with the ERK antisense oligonucleotide did not restore PDGF-treated cell adhesion in the presence of 50 μM GRGDS peptide (Fig. 4B). When concentration of GRGDS peptide was increased from 50 to 200 μM, the adhesion of cells treated with the transfectant reagent alone and with ERK control oligonucleotide decreased by 36 ± 2 and 41 ± 6%, respectively. In contrast, this decrease was reduced to 11 ± 7% (p < 0.01) when SMC were treated with the ERK antisense oligonucleotide (Fig. 4C). This indicates that the decrease in ERK levels is associated with the reduced modulation of integrin avidity by PDGF and suggests that the PDGF-induced ERK pathway reduces integrin avidity. Moreover, in accordance with results obtained with PD98059 (Fig. 3B), ERK depletion partially restored the adhesion of PDGF-treated cells to 0.5 μg/ml fibronectin but did not restore the adhesion to 5 μg/ml fibronectin (Fig. 4D). Residual ERK expression (30%) might be sufficient to modulate the avidity of integrins expressed in the lowest avidity state and, thus, to induce an inhibition of cell adhesion in the presence of 50 μM GRGDS peptide. Thus, our results suggest that PDGF might modulate SMC adhesion by two distinct mechanisms, 1) the ERK-dependent mechanism, which decreases integrin avidity and adhesion at low fibronectin-coating concentrations, and 2) the ERK-independent pathway, which does not affect integrin avidity but decreases adhesion at all fibronectin concentrations.

*PDGF Inhibits Focal Adhesion Assembly and Stress Fiber Formation—Mechanisms other than the regulation of ligand binding may also be involved in the modulation of cell adhesion. In particular, the organization of the cytoskeleton may affect the interaction between the cell surface and the extracellular matrix (8). We compared the spreading efficiency of control and PDGF-treated cells after plating on fibronectin for 60 min. PDGF reduced the number of spread cells (Fig. 5A), from 74 ± 5 to 48 ± 7% (p < 0.001). This suggests that the ERK-independent mechanism involved in PDGF-induced inhibition of cell adhesion might affect the organization of the cytoskeleton. To investigate this hypothesis we examined the formation of stress fibers and focal adhesions in control and PDGF-treated cells. SMC were plated onto fibronectin and stained for F-actin and paxillin. After 30 min, F-actin was present in membrane protrusions (Fig. 5B, a). Cytoplasmic actin filaments were visualized after 60 min (Fig. 5B, b), and numerous stress fibers were present after 2 h (Fig. 5B, c). In contrast, only a few PDGF-treated cells had begun to spread by 30 min (Fig. 5C, a). F-actin staining was visualized in the lamellipodia after 60 min, but cytoplasmic actin filaments were not detected (Fig. 5C, b). Moreover, few actin filaments were visible after 2 h (Fig. 5C, c).

To determine whether the inhibition of stress fiber formation was accompanied by changes in focal adhesion assembly, we examined the localization of paxillin during cell spreading. Paxillin punctuated the periphery of control cells 30 min after plating (Fig. 5B, d). These structures, known as focal complexes...
Data represent the mean ± S.E. of four independent experiments. Control SMC (B) or PDGF-treated SMC (C) (4 ng/ml) were plated on fibronectin-coated coverslips (5 μg/ml) for 30 min (a and d), 60 min (b and e), or 120 min (c and f) and stained for F-actin (green, a–c) and paxillin (red, d–f). Representative results from four independent experiments are shown.

(14), increased in size, and typical focal adhesion appeared after 2 h (Fig. 5B, e and f). In PDGF-treated cells, paxillin staining was localized in focal complexes at the cell periphery after 60 min (Fig. 5C, e). Characteristic focal adhesions were visible after 2 h (Fig. 5C, f), but the size and/or number of focal adhesion was lower than in the control cells. These results indicate that PDGF delays focal complex formation and inhibits stress fiber assembly.

PDGF Delays Tyrosine Phosphorylation of Paxillin—To confirm that PDGF inhibits initial focal complex formation, we investigated the tyrosine phosphorylation state of paxillin, which is known to be associated with focal adhesion assembly (24). Immunoprecipitation of paxillin followed by a Western blotting with an anti-phosphotyrosine antibody revealed that tyrosine phosphorylation of paxillin could be detected after 30 min and reached a maximum after 60 min (Fig. 6). Pretreatment of SMC with PDGF delayed tyrosine phosphorylation of paxillin, which appeared after 60 min and reached the same level as control cells after 120 min. In conclusion, pretreatment of SMC with PDGF delays tyrosine phosphorylation of paxillin, which correlates with the delay in focal complex formation.

Src Family Tyrosine Kinases Are Not Inhibited by PDGF—Different tyrosine kinases, such as focal adhesion kinase and/or Src, are activated by integrin engagement and are involved in tyrosine phosphorylation of paxillin in response to integrin-matrix interactions. Because SMC adhesion did not induce focal adhesion kinase tyrosine phosphorylation even though it is recruited at focal adhesions (results not shown), we examined the involvement of Src kinase(s) in the tyrosine phosphorylation of paxillin. The tyrosine phosphorylation of paxillin was completely abolished in the presence of 5 μM PP1, an Src family-selective tyrosine kinase inhibitor (Fig. 7A).

We subsequently determined whether Src kinases are a target of PDGF action. Kinase activity was assessed in the presence and absence of PDGF after immunoprecipitation of Src kinases(s) followed by phosphorylation of enolase substrate. In the absence of PDGF, Src kinase activity was transient, reaching maximal levels (3–4-fold increase) after 10–15 min and then returning to basal levels by 20 min (Fig. 7B). Src kinase activity was not substantially altered by the presence of PDGF.

These results suggest that the activation of the Src kinase is not dependent on focal complex formation. Delayed tyrosine phosphorylation of paxillin in response to PDGF is not due to an inhibition of Src family tyrosine kinase activity.

DISCUSSION

The adhesion of the cells to the extracellular matrix involves interactions between integrins and ligands, formation of focal adhesions, and cell spreading (8). We showed that PDGF inhibits the adhesion of SMC to fibronectin by interfering with these processes.

PDGF dramatically inhibited SMC adhesion (80%) in low fibronectin concentrations (0.5 μg/ml). This occurred by ERK-dependent and -independent pathways, as shown by the partial restoration of adhesion in the presence of a specific inhibitor of MEK (mitogen-activated protein kinase/ERK kinase) activation, PD98059. Moreover, the remaining inhibition (50%) was unaffected by the fibronectin concentration. These results suggest that ERK activation decreases integrin avidity (i.e., binding to multivalent ligand). The ability of the GRGDS peptide (0–200 μM) to prevent integrin-mediated cell adhesion was increased in cells that had been treated with PDGF, indicating a decrease in integrin avidity. Adhesion of SMC to fibronectin was inhibited by 80% in the presence of the GRGDS peptide (2 mM). This suggests that this adhesion is mainly mediated by α5 and α5β1 integrins (25). In SMC, α5β1 integrin is the major fibronectin receptor (26, 27), but significant amounts of α5β3 integrin are also present in porcine aortic SMC (26). However, consistent with previous studies (28), the anti-α5β3 monoclonal antibody did not affect SMC adhesion (data not shown), indicating that the α5β3 integrin is not involved in SMC adhesion to fibronectin and suggesting that PDGF regulates the α5β3 integrin expressed in the lowest avidity states in SMCs. We confirmed that ERK is involved in the PDGF-mediated decrease in avidity of integrins by use of an oligonucleotide that targets the ERK1 and ERK2 translation initiation sites. The enhanced efficiency of the GRGDS peptide to inhibit the adhesion of PDGF-treated cells was significantly reduced after depletion of ERKs. Residual ERK protein (90%) might explain the partial restoration of integrin avidity; in particular, the inability of the antisense oligonucleotide to restore cell adhesion in the presence of 50 μM GRGDS peptide. ERK levels might be sufficient to modulate these integrins when they are in a low avidity state. However, we cannot rule out the possibility that other pathways are involved. Our results suggest that growth factors can modulate integrin activation via an ERK-dependent signaling pathway in physiological conditions.
Fig. 6. Effects of PDGF on tyrosine phosphorylation of paxillin. Control and PDGF-treated SMC (4 ng/ml) were plated out in dishes coated with fibronectin (5 μg/ml) for 0, 30, 60, or 120 min. Cell lysates were immunoprecipitated (IP) with an anti-paxillin (Pax) antibody and immunoblotted (WB) with an anti-phosphotyrosine (P-Tyr) antibody. Blots were reprobed with an anti-paxillin antibody to ensure equal loading. Representative results from three independent experiments are shown.

Fig. 7. Effects of PDGF on the adhesion-induced activity of Src family tyrosine kinases. A, involvement of Src tyrosine kinases in paxillin tyrosine phosphorylation. Suspended SMC were incubated for 30 min with various concentrations of PP1 and plated in dishes coated with fibronectin (5 μg/ml) for 45 min. Cell lysates were immunoprecipitated (IP) with an anti-paxillin (Pax) antibody and immunoblotted (WB) with an anti-phosphotyrosine (P-Tyr) antibody. B, control or PDGF-treated SMC (4 ng/ml) were plated in dishes coated with fibronectin (5 μg/ml) for 0, 5, 10, 15, or 20 min. Src tyrosine kinase activity was determined using the enolase assay as described under "Experimental Procedures." C, enolase phosphorylation was quantified by scanning densitometry, and the increase in Src activity was calculated for control cells (white bars) and PDGF-treated cells (shaded bars). Representative results from four independent experiments are shown.

The activation of integrins involves enhanced affinity and/or diffusion-regulated clustering (9, 10), however adhesion assays cannot distinguish between these two mechanisms. Our observations are consistent with data showing that the activation of the ERK pathway suppresses the activation of the chimeric integrins expressed in Chinese hamster ovaries (12). They also indicate that the modulation of integrin activation might be involved in the Ras signaling pathways required for PDGF-directed migration (17).

Suppression of integrin activation by overexpressing active c-Raf inhibits matrix fibronectin assembly and results in rounded cells (12). However, in this study the formation of focal adhesions and stress fibers was not examined. In our model, PDGF induces the inhibition of SMC adhesion to high fibronectin concentrations independently of the ERK pathway. Moreover, this inhibition correlates with an inhibition of focal complex formation, which was still observed after ERK inhibition by PD98059 (result not shown). These small adhesive structures are found at the periphery of the cell in the early stages of cell spreading (29, 30) and develop into large focal adhesions. Their assembly is independent of small GTPase Rac-induced lamellipodia formation (31) and of small GTPase Rho activity (29). The clustering of integrins into focal complexes induced by the small GTPases Rac and Cdc42 has not been characterized, but these structures contain the same protein constituents as focal adhesions (14).

Two mechanisms might be involved in the inhibition of focal complex assembly. First, assembly may be blocked by disruption of integrin clustering. However, adhesion was still inhibited when integrin avidity was restored by ERK depletion or ERK inhibition. Moreover, the activation (32) and focal complex localization (13) of Src family tyrosine kinases only require clustering of integrin. In our model, activation of Src family tyrosine kinases was not inhibited in PDGF-treated SMC. This suggests that PDGF does not inhibit integrin clustering.

The second possibility is that cytoskeletal protein interactions and/or protein recruitment are inhibited. It has been proposed that focal adhesion assembly is regulated by paxillin (33, 34), which is a focal adhesion adaptor protein that recruits structural and signaling molecules through its N terminus and is targeted to focal adhesions through its C terminus (33). Associated kinases mediate the serine and threonine phosphorylation of the LIM domains of the C terminus (35) of paxillin. This phosphorylation modulates the focal adhesion localization rate of paxillin, which is correlated with the cell capacity to adhere to fibronectin. It remains to be determined whether PDGF can modulate the adhesion-mediated phosphorylation of LIM domains. Disassembly of focal adhesions mediated by growth factors precedes cell migration and reduces cell-substratum adhesiveness (15, 36). The signaling pathway involved in this process is still controversial. Recent reports (36, 37) indicate that focal adhesion disassembly induced by the epidermal growth factor is mediated by the protease calpain, which is activated by the ERK pathway. Loss of focal adhesions induced by treating SMC with degraded collagen is also mediated by calpain proteolysis of focal adhesion kinase, paxillin, and talin.
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(38). However, we did not observe cleavage of focal adhesion kinase in PDGF-treated cells (results not shown). Finally, a Ras-dependent but ERK-independent pathway has been proposed to be involved in focal adhesion disassembly (15). The effects of PDGF treatment on SMC adhesion might reflect this disorganization of focal adhesion.

The small GTPase, Rho, controls focal adhesion assembly and stress fiber formation by stimulating actomyosin-based contractility (39). Several studies show that Rho activity is down-regulated by Rac (40, 41), which can be induced by PDGF (41). Rho also controls the tyrosine phosphorylation of proteins (29, 42), which is not required for focal adhesion assembly (43), and its downstream effector, a member of the Wiskott-Aldrich syndrome protein family, N-WASP, were recently shown to be activated by PDGF (47). Activation of this pathway depends on the p85α regulatory subunit of phosphoinositide 3-kinase and is required for PDGF-induced NII3T3 cell migration on collagen. Moreover, this pathway also mediates the disassembly of stress fiber and the reduction of focal adhesions. Consistent with our results, the formation of actin stress fibers induced by a constitutively active mutant, v-Rho, was inhibited by the expression of p85α.

In summary we have shown that PDGF might regulate the adhesion of SMC to fibronectin, a process required for cell migration. PDGF regulates two of the steps involved in cell adhesion, integrin-matrix interaction, by decreasing integrin avidity, and the formation of focal complexes.

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