Journal of Cellular Biochemistry 99:1553–1563 (2006)

Differential Effects of Imatinib on PDGF-Induced Proliferation and PDGF Receptor Signaling in Human Arterial and Venous Smooth Muscle Cells

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Abstract Platelet-derived growth factor (PDGF) has been implicated in smooth muscle cell (SMC) proliferation, a key event in the development of myointimal hyperplasia in vascular grafts. Recent evidence suggests that the PDGF receptor (PDGFR) tyrosine kinase inhibitor, imatinib, can prevent arterial proliferative diseases. Because hyperplasia is far more common at the venous anastomosis than the arterial anastomosis in vascular grafts, we investigated whether imatinib also inhibited venous SMC (VSMC) proliferation, and examined possible differences in its mechanism of action between VSMC and arterial SMC (ASMC). Human ASMC and VSMC were stimulated with PDGF-AB, in the presence or absence of imatinib (0.1–10 μM). Proliferation was assayed using the 5-bromo-2′-deoxyuridine (BrdU) incorporation assay, while PDGFR, Akt and ERK1/2-mitogen activated protein kinase (MAPK) signaling pathways were investigated by immunoblotting. The proliferative response to PDGF at 50 and 100 ng/ml was 32 and 43% greater, respectively, in VSMC than in ASMC. Similarly, PDGF-stimulated proliferation was more sensitive to inhibition by imatinib in VSMC than ASMC ($IC_{50} = 0.05$ μM vs. 0.4 μM; $P < 0.01$). Imatinib also more effectively inhibited PDGF-induced phosphorylation of PDGFRβ and Akt in VSMC, compared to ASMC. These data highlight inherent pharmacodynamic differences between VSMC and ASMC in receptor and cell signaling functions and suggest that imatinib therapy may be useful for the prevention of venous stenosis in vascular grafts. J. Cell. Biochem. 99: 1553–1563, 2006.

Key words: venous smooth muscle cell; vascular graft; platelet-derived growth factor; Akt; receptor tyrosine kinase inhibitor

Bypass grafts often fail as a result of the development of myointimal hyperplasia and stenosis induced by surgical trauma and hemodynamic changes [Liu, 1998; Huynh et al., 1999]. Clinically, venous grafts are more prone to stenosis than arterial grafts [Cameron et al., 1996]. Similarly, myointimal hyperplasia develops much more frequently at the venous anastomosis than at the arterial anastomosis of the arteriovenous grafts used for hemodialysis [Kelly et al., 2002]. In addition to hemodynamic factors, differences in intrinsic cellular behaviors between arterial smooth muscle cells (SMCs) and venous SMC may explain in part the differences in the propensity of arteries and veins to stenosis. Despite the increased susceptibility of the venous anastomosis to myointimal hyperplasia, most pathophysiology and drug studies have been performed in arterial SMC. Investigations on the differences in drug susceptibility between arterial and venous SMC are scarce.

Growth factors, particularly platelet-derived growth factor (PDGF), released from activated platelets, SMC and monocytes are important mediators of SMC proliferation [Heldin and Westermark, 1999]. Sirois et al. [1997] demonstrated that inhibition of PDGF receptor β

Grant sponsor: National Heart, Lung and Blood Institute; Grant number: R01-HL67646; Grant sponsor: The Merit Review Program of the Department of Veterans Affairs; Grant sponsor: The Dialysis Research Foundation; Grant sponsor: The National Kidney Foundation of Utah & Idaho.

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Received 3 March 2006; Accepted 19 April 2006
DOI 10.1002/jcb.20993

(PDGFRβ) by antisense oligonucleotides suppressed intimal thickening in the rat carotid artery following balloon injury. Administration of antibodies to both PDGF Rα and PDGFRβ was reported to induce intimal atrophy in a baboon graft model [Englesbe et al., 2004]. These observations support the pathogenic role of PDGF and PDGFRs in proliferative vascular diseases and their potential as molecular targets for drug therapy to prevent myointimal hyperplasia.

Imatinib mesylate (Gleevec or STI571) is a tyrosine kinase inhibitor with specificity towards c-kit, c-Abl, and PDGFR [Druker, 2002]. By inhibiting the tyrosine kinase activity of Bcr-Abl [Druker et al., 1996], imatinib has been very effective in the treatment of chronic myelogenous leukemia. Similarly, by inhibiting c-kit tyrosine kinase, imatinib induces regression of gastrointestinal stromal tumors [Heinrich et al., 2002]. The therapeutic efficacy of imatinib has also been demonstrated in animal models of arterial proliferative disease, including restenosis after balloon angioplasty [Myllarniemi et al., 1999], diet-induced atherosclerosis [Boucher et al., 2003], and transplant atherosclerosis [Nykanen et al., 2005]. Most studies on the anti-proliferative effects of imatinib, however, have employed rat or bovine aortic SMC or transformed cancer cell lines. In the present study, we compared normal arterial and venous SMC responses to PDGF and discovered interesting differences in PDGF-induced growth responses between these two cell types that were consistent in both human and porcine cells. These differences may explain their different propensities to proliferate in arteriovenous hemodialysis grafts and the increased stenosis rates in venous bypass grafts. We also examined the efficacy and mechanisms by which imatinib might inhibit human venous SMC. Noteworthy differences in imatinib effects on key proliferative signaling pathways were observed between these two vascular SMC types.

METHODS

Reagents and Antibodies

All cell culture reagents were purchased from Cascade Biologics (Portland, OR), except fetal bovine serum (FBS), which was from Atlanta Biologicals (Lawrenceville, GA). Imatinib mesylate (Gleevec, STI 571) was obtained from Novartis Pharmaceuticals (Basel, Switzerland), dissolved in dimethyl sulfoxide (DMSO) to generate a stock solution of 10 mM and stored at 4°C until use. The stock solution was diluted with cell culture medium to the appropriate concentrations and immediately added to the cells for each experiment. Recombinant PDGF-AB was purchased from R&D Systems (Minneapolis, MN). LY294002 and PD98059 were purchased from Cell Signaling Technologies (Beverly, MA). Polyclonal antibodies directed against PDGFRβ (sc-339), phospho-PDGFRβ (sc-12909R) and goat anti-rabbit antibody conjugated with horse-radish peroxidase (HRP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific for ERK1/2, phospho-ERK1/2, Akt and phospho-Akt (9102, 9101, 9272, and 9271, respectively) were obtained from Cell Signaling Technologies. Precast polyacrylamide gels, nitrocellulose membranes and Immun-Star HRP Chemiluminescent kit were purchased from Bio-Rad Laboratories (Hercules, CA). Complete Mini-Protease Inhibitor Cocktail was obtained from Roche Applied Science (Indianapolis, IN). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical (Rockford, IL).

Cell Culture

Human aortic SMC (ASMC) and saphenous vein SMC (VSMC) were purchased from Cambrex Bio Science (Chicago, IL). All cells were cultured in a humidified 37°C incubator with 5% CO_2 in Medium 231, 20% FBS and smooth muscle growth supplement (SMGS) with 5 μg/ml insulin, 0.5 ng/ml human epidermal growth factor, 2 ng/ml human basic fibroblast growth factor, 100 μg/ml streptomycin, 100 U/ml penicillin G and 0.25 μg/ml amphotericin B. For all experiments, both ASMC and VSMC from three different donors and from passages 3–7 were used. Porcine SMC were collected from femoral arteries and veins of normal Yorkshire cross domestic swine by collagenase and elastase digestion after mechanical removal of the endothelial and adventitial layers as previously described [Brar et al., 2002]. Cells were grown to 70–80% confluence and then rendered quiescent by incubation with medium containing 0.5% FBS but no additional growth factors. The cells were kept quiescent for 48 h before the addition of PDGF and/or imatinib for proliferation experiments.

Cell Proliferation

SMC were seeded on 96-well microtiter plates at a density of 1×10^4 cells/well. Various concentrations of PDGF-AB were added to the culture medium and DNA synthesis was assessed by 5-bromo-2′-deoxyuridine (BrdU) incorporation (Cell Proliferation Biotrak ELISA System, Amersham Biosciences, Piscataway, NJ) after 48 h. All experiments were performed in triplicate wells and repeated in separate experiments. In other experiments, SMC (1×10^4 cells/well) were incubated in 96-well plates with various concentrations of imatinib for 90 min and then stimulated with 50 ng/ml of PDGF-AB. A 48-h proliferation assay was then performed using the BrdU incorporation assay. Briefly, after 48 h of incubation with PDGF, BrdU labeling solution (BrdU concentration: 10 μM) was added to the cells and incubated for another 12–24 h. After removing the culture medium, the cells were fixed and the DNA denatured by the addition of a fixative. After a 30-min incubation at room temperature, the fixative was removed. Subsequently, peroxidase-labeled anti-BrdU monoclonal antibody was added and the plate incubated at room temperature for 90 min. The BrdU-antibody complexes were detected by a colorimetric reaction with the substrate (3, 3′, 5, 5′-tetramethylbenzidine), with the optical density read at 450 nm in a microplate reader (Multiskan Ascent, Thermo Electron Corporation, San Jose, CA).

In some experiments, the cells were pretreated with various concentrations of LY 294002 (an inhibitor of phosphoinositide-3-kinase (PI3K)) or PD98059 (an inhibitor of ERK activation) for 60 min prior to PDGF stimulation. The 48-h BrdU incorporation assay was performed to assess the effects on proliferation.

Western Blotting

Serum-starved SMC were pretreated with various concentrations of imatinib for 90 min and then stimulated with 50 ng/ml of PDGF-AB for 10 min. After PDGF stimulation, the cells were washed twice with ice-cold PBS and lysed in a lysis buffer (PBS containing 0.6% NP-40, 150 mM NaCl, 10 mM HEPES, pH 7.9, mixed with one tablet of protease inhibitor cocktail per 10 ml buffer immediately before use). The lysates were incubated on ice for 30 min and then centrifuged at 10,000*g* for 10 min. Protein concentrations in the cell lysates were determined using the BCA protein assay. Samples containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis on precast 4–20% gradient gels. Following electrophoresis and transfer to nitrocellulose membranes, the blots were incubated for 1 h at room temperature with antibodies to PDGFRβ and phospho-PDGFRβ, or overnight at 4°C with antibodies to ERK1/2, phospho-ERK1/2, Akt, and phospho-Akt. Immunoblot detection was performed using an Immun-Star HRP Chemiluminescent kit according to the manufacturer's instructions. Western blot band intensity was quantitated using the NIH ImageJ software (http://rsb.info.nih.gov/ij/).

Flow Cytometry

For flow cytometry experiments, quiescent ASMC and VSMC were treated with 50 ng/mL of PDGF-AB in the presence or absence of various concentrations of imatinib as described above. After 48 h, the cells were detached from the plate using trypsin, washed with PBS, then fixed in 70% ethanol for 30 min on ice. The cells were then washed twice with PBS and resuspended in a solution containing RNase (1 mg/ml) and propidium iodide (50 μg/ml). The samples were incubated in the dark at room temperature for 20 min and subjected to fluorescence-activated cell sorter (FACS) analysis. Cell cycle profiles were determined using a CellQuest system (Becton-Dickinson, San Jose, CA). The percentage of cells in the G_0/G_1, S or G_2-M phase was determined by analyzing the FACS histograms using the ModFitLT software (Verity Software, Topsham, ME).

Statistical Analysis

Results are reported as mean ± SD. Comparisons between ASMC and VSMC were performed using a repeated measures analysis of variance (ANOVA); further comparisons between these cells at specific concentrations were performed using a paired Student's *t*-test. Comparisons between different concentrations and a single control group (normalized to 100%) were performed using the one sample *t*-test. Comparisons of cell cycle distribution of SMCs were first analyzed using one-way ANOVA; further comparisons among the different experimental conditions were performed using an unpaired Student's *t*-test. All statistical

analyses were performed using the StatView (version 4.57) program. Statistically significant results are defined as $P < 0.05$.

RESULTS

Different Sensitivity of ASMC and VSMC Proliferation to PDGF and Imatinib

The PDGF isoform PDGF-AB was used throughout this study because of its known ability to stimulate both α and β PDGFR subtypes, and because of its robust mitogenic and chemotactic effects on cells expressing both α and β PDGFRs [Heldin and Westermark, 1999]. In time-course studies, we found that the growth effects of PDGF on human SMC were sustained through 72 h but were maximal by 48 h (data not shown). Thus the dose-response of PDGF-stimulated proliferation of SMC was determined at 48 h. As shown in Figure 1A, increasing concentrations of PDGF led to increased incorporation of BrdU in both cell types. The maximal effect of PDGF on VSMC proliferation was achieved at 200 ng/ml (data not shown). The proliferative response to PDGF at 50 and 100 ng/ml was 32% and 43% greater, respectively, in VSMC than in ASMC. Similar differences were also observed between porcine VSMC and ASMC indicating these differences in proliferative response are probably not species-specific (data not shown).

To determine the effects of imatinib on proliferation, cells were stimulated with PDGF (50 ng/ml) in the presence of incremental concentrations of imatinib. This concentration of PDGF is in the same range used in other PDGF-stimulated cell proliferation studies and in the range of serum concentrations of a variety of patient populations [Myllarniemi et al., 1997; Rossi et al., 1998; Cianciolo et al., 1999; Servant et al., 2000; Gilbert et al., 2001]. Proliferation of both ASMC and VSMC was inhibited by imatinib in a dose-dependent manner (Fig. 1B), with statistically significant effects observed at 0.3 μM for ASMC ($P < 0.001$) and at 0.03 μM for VSMC ($P < 0.001$). The inhibitory effect of imatinib on VSMC was significantly greater than on ASMC ($P < 0.001$). The concentration of imatinib required to inhibit proliferation by 50% (IC_{50}) was 0.05 μM for VSMC, which was eightfold lower than ASMC (0.4 μM). Thus, both types of SMC were responsive to imatinib, with VSMC being more sensitive.

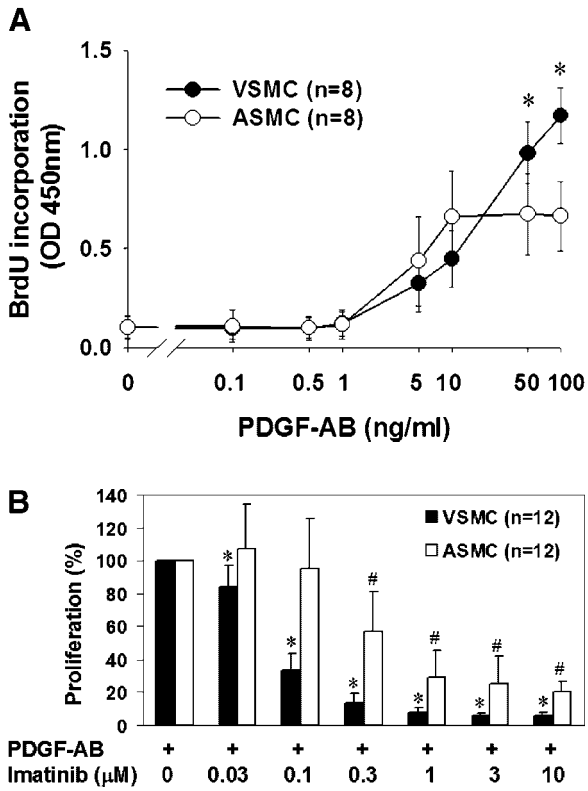

Fig. 1. Effect of PDGF-AB and imatinib on BrdU incorporation in SMC. **A**: Venous SMC (VSMC; closed circles) and arterial SMC (ASMC; open circles) were serum-starved for 48 h and then treated with incremental concentrations of PDGF-AB. After an additional 48 h, BrdU incorporation was assessed. Results represent means ± SD of eight experiments. PDGF increased BrdU incorporation in both cell types in a dose-dependent manner. $^{*}P < 0.0001$, VSMC versus ASMC as determined by repeated measures ANOVA. **B**: Subconfluent and quiescent venous (VSMC, closed bars) and arterial SMC (ASMC, open bars) were pretreated with incremental concentrations of imatinib for 90 min, then stimulated with 50 ng/ml PDGF-AB. Proliferation was assessed by BrdU incorporation after 48 h. Percent proliferation was calculated by comparison with cell proliferation stimulated by PDGF alone. Results represent means ± SD of 12 experiments. $^{*}P < 0.05$ versus PDGF alone in VSMC; $^{\#}P < 0.05$ versus PDGF alone in ASMC. The inhibitory effect of imatinib was significantly greater in VSMC than in ASMC.

Different Effect of Imatinib on Autophosphorylation of PDGFRβ in ASMC and VSMC

Imatinib was tested for its ability to inhibit the autophosphorylation of the PDGFRβ. Serum-starved ASMC and VSMC were treated with various concentrations (0.01–10 μM) of imatinib for 90 min and then stimulated with 50 ng/ml PDGF-AB for 10 min. The cell lysates were subjected to Western blotting with anti-PDGFRβ or anti-phosphorylated PDGFRβ as

primary antibody. The results clearly demonstrated that PDGF induced PDGFRβ autophosphorylation, which was inhibited by imatinib in both cell types. However, the phosphorylation of PDGFRβ in VSMC was almost completely inhibited by 0.1 μM imatinib (Fig. 2), whereas approximately tenfold higher concentrations of imatinib were necessary to inhibit the phosphorylation of PDGFRβ in ASMC to a similar extent. This difference in phosphorylation of PDGFRβ in response to imatinib between ASMC and VSMC is consistent with the difference in IC_{50} values observed in the cell proliferation assays (Fig. 1B). The total amount of PDGFRβ protein did not change significantly after this short exposure to imatinib (Fig. 2).

Effects of Intracellular Signaling Inhibitors on PDGF-Stimulated SMC Proliferation

To elucidate the relative roles of the intracellular signaling pathways, PI3K and ERK1/2-mitogen activated protein kinase (MAPK), in mediating PDGF-AB-stimulated SMC proliferation, we investigated the effects of LY294002, a specific inhibitor of PI3K, and PD98059, a specific inhibitor of ERK1/2 activation. Treatment with LY294002 significantly decreased PDGF-induced proliferation in a concentration-dependent manner in both human ASMC and VSMC (Fig. 3), with no significant differences in concentration-dependence or maximal response between the cell types. While PD98059 also inhibited the proliferation of both cell types, the inhibition was relatively modest in ASMC ($46.8 \pm 5.5\%$) even at the highest concentration tested (50 μM; Fig. 3). In contrast, PD98059 inhibited VSMC by $80.8 \pm 10.1\%$ at the same concentration (50 μM). These findings suggest that both the PI3K pathway and ERK1/2-MAPK pathway are important mediators of PDGF-induced proliferation in VSMC. However, the contribution of the ERK1/2-MAPK pathway appears to be less important than that of the PI3K pathway in PDGF-stimulated ASMC proliferation.

Effect of Imatinib on Phosphorylation of Akt and ERK1/2

Since the PI3K and ERK1/2-MAPK pathways appear to mediate the PDGF-induced proliferation of VSMC and ASMC to various extents, the effect of imatinib on the activation of these pathways was investigated in PDGF-stimulated cells. The results clearly demonstrated that PDGF-induced Akt phosphorylation was completely inhibited by 10 μM imatinib in both VSMC and ASMC (Fig. 4). A significant reduction in the phosphorylation of Akt was found at 1 μM imatinib in VSMC, but no significant effect was observed at the same concentration of imatinib in ASMC (Fig. 4). In addition, at concentrations of imatinib that almost completely blocked the autophosphorylation of the

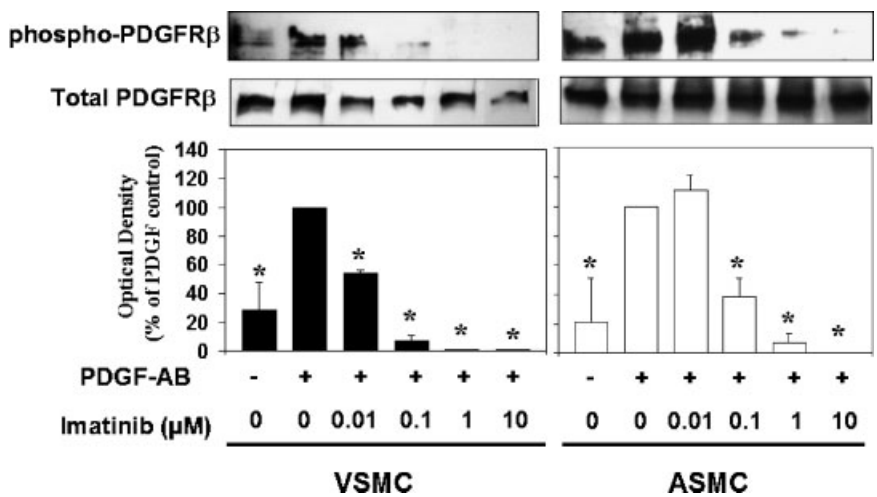

Fig. 2. Effect of imatinib on PDGF-induced phosphorylation of PDGFRβ. Subconfluent and quiescent VSMC and ASMC were pretreated with incremental concentrations of imatinib for 90 min, and then stimulated with 50 ng/ml of PDGF-AB for 10 min. Cell lysates were subjected to immunoblotting with specific antibodies for either phosphorylated PDGFRβ (phospho-PDGFRβ) or total PDGFRβ. Representative blots are shown on the top, while the mean (±SD) intensity of the bands from three separate experiments are presented as bars in the bottom panels for VSMC (**left panel**) and ASMC (**right panel**) respectively. $^*P < 0.05$; PDGF + imatinib versus. PDGF alone.

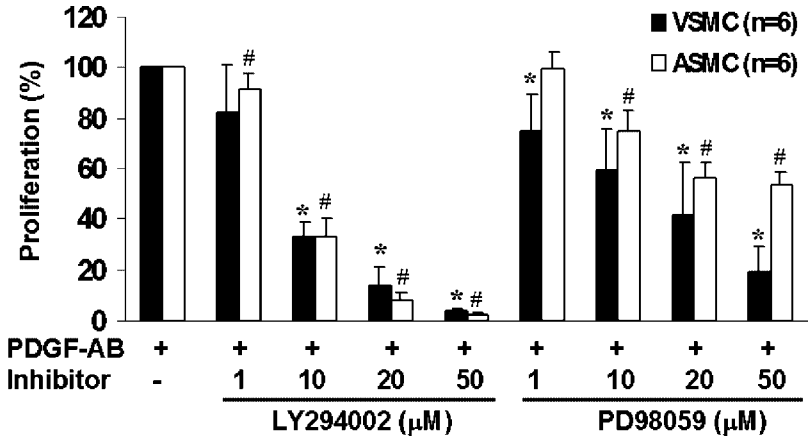

Fig. 3. Effects of signaling inhibitors on PDGF-stimulated SMC proliferation. Subconfluent and quiescent venous (VSMC, closed bars) and arterial SMC (ASMC, open bars) were pretreated with incremental concentrations of either LY294002 (PI3K inhibitor) or PD98059 (ERK1/2 inhibitor) for 90 min. The proliferation stimulated by PDGF-AB (50 ng/ml) in the presence or absence of the inhibitors was examined using BrdU incorporation assay after 48 h. The proliferation stimulated by PDGF alone was arbitrarily defined as 100%. Results represent means ± SD of six separate experiments. $^{*,\#}P < 0.05$; PDGF + inhibitor versus PDGF alone in the corresponding cell types.

PDGFβ receptor (0.1 μM in VSMC and 1 μM in ASMC; Fig. 2), Akt phosphorylation was essentially unaffected. The total amount of Akt did not change significantly after this short exposure to imatinib (Fig. 4).

The effect of imatinib on the phosphorylation of ERK1/2 was also examined. In contrast to the complete inhibition of Akt phosphorylation, only partial inhibition of ERK1/2 phosphorylation was observed even at 10 μM imatinib (Fig. 5), despite complete inhibition of PDGFR autophosphorylation. The total amount of ERK1/2 did not change significantly after this short exposure to imatinib (Fig. 5).

Imatinib Inhibits G_1 to S Cell Cycle Progression in SMC

To further clarify the cellular mechanism underlying growth inhibition in the presence of imatinib, cell cycle profiles were analyzed by flow cytometry 48 h after treatment with PDGF, with and without imatinib. Figure 6 shows that essentially all unstimulated SMC (negative

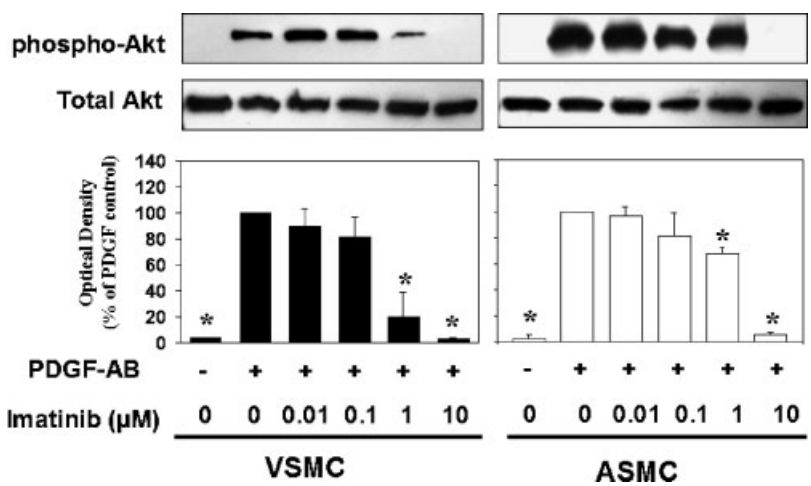

Fig. 4. Effect of imatinib on PDGF-induced phosphorylation of Akt. Subconfluent and quiescent venous (VSMC, closed bars) and arterial SMC (ASMC, closed bars) were pretreated with various concentrations of imatinib for 90 min, and then stimulated with 50 ng/ml of PDGF-AB for 10 min. Cell lysates were subjected to immunoblotting using specific antibodies for either phosphorylated Akt (phospho-Akt) or total Akt. Representative blots are shown on the top, while the mean (±SD) intensity of the bands from three separate experiments are presented as bars in the bottom panels for VSMC (**left panel**) and ASMC (**right panel**) respectively. $^{*}P < 0.05$; PDGF + imatinib versus PDGF alone.

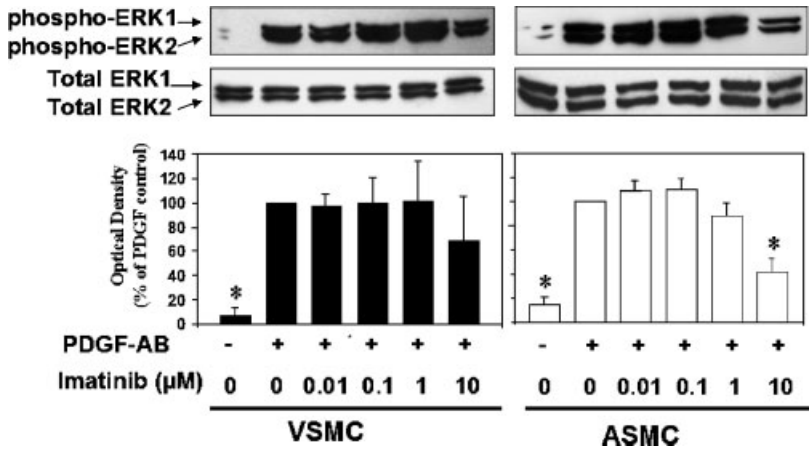

Fig. 5. Effect of imatinib on PDGF-induced phosphorylation of ERK1/2. Subconfluent and quiescent venous (VSMC, closed bars) and arterial SMC (ASMC, open bars) were pretreated with various concentrations of imatinib for 90 min, then stimulated with 50 ng/ml of PDGF-AB for 10 min. Cell lysates were subjected to immunoblotting using specific antibodies for phosphorylated ERK1 (phospho-ERK1) or ERK2 (phospho-ERK2) or total ERK1 or ERK2. Representative blots are shown on the top, while the mean (±SD) intensity of the bands from three separate experiments are presented as bars in the bottom panels for VSMC (**left panel**) and ASMC (**right panel**) respectively. $^{*}P < 0.05$; PDGF + imatinib versus PDGF alone.

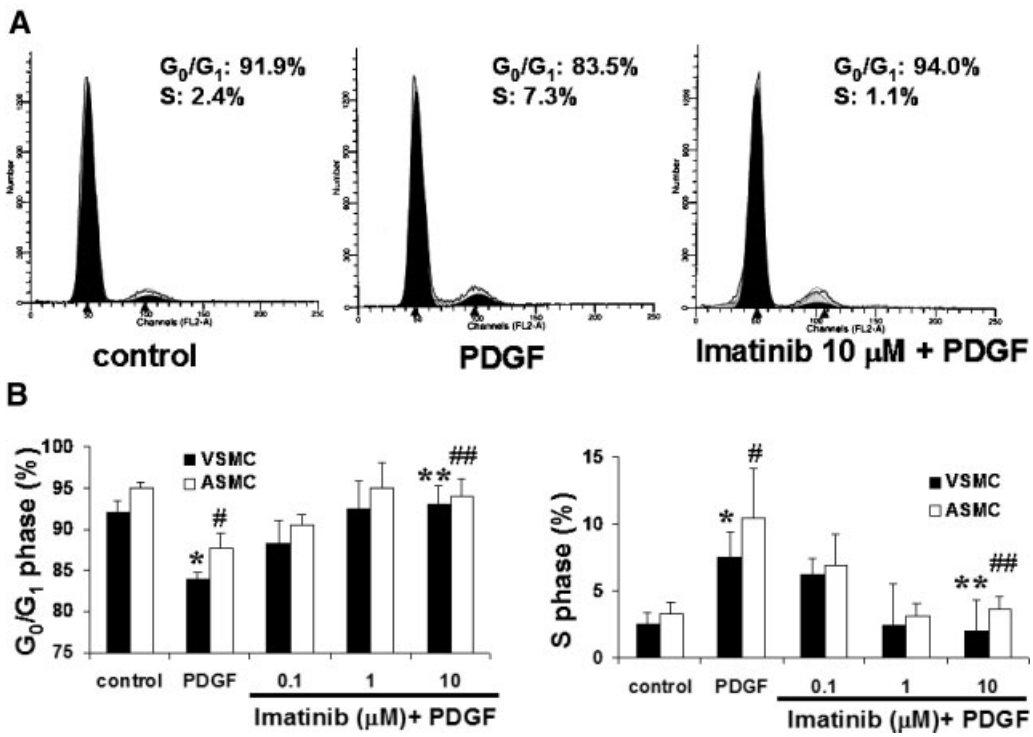

Fig. 6. Effects of imatinib on cell cycle distribution of PDGF-stimulated SMC. Venous (VSMC, closed bars) or arterial (ASMC, open bars) SMC were treated with 50 ng/mL PDGF-AB and various concentrations of imatinib. After 48 h, the cells were subjected to fluorescence-activated cell sorter (FACS) analysis. SMC without PDGF or imatinib served as controls. **A**: Representative DNA histograms. **B**: Percentage of cells in the G_0/G_1 (**left panel**) or S phase (**right panel**). Each bar represents the mean ± SD of three separate experiments. $^{*,\#}P < 0.05$; PDGF versus control and $^{**,\#\#}P < 0.05$; imatinib + PDGF versus PDGF alone in the corresponding cell types. PDGF decreased the percentage of cells in the G_0/G_1 phase while increasing the percentage of cells in the S phase. These changes were reversed by imatinib.

control) were in the G_0/G_1 phase of the cell cycle. PDGF stimulated DNA synthesis and progression into the S phase. This effect of PDGF was inhibited by imatinib in a dose-dependent manner with cells being primarily in G_0/G_1 at high doses of imatinib.

DISCUSSION

Although the venous anastomosis is more prone to myointimal hyperplasia than the arterial anastomosis associated with vascular grafts, there is a paucity of data on the effects of PDGF and PDGF receptor inhibition on VSMC. In the present study, we have found that VSMC demonstrate a greater PDGF-induced proliferative response and differences in the activation of downstream signaling pathways than ASMC. These findings might not only explain the higher incidence of venous stenosis in AV grafts, but also provide important insights into possible therapeutic strategies to prevent graft stenosis.

There are only a few reports in the literature of differences between arterial and venous SMC with regard to cell growth. Co-culture studies of endothelial cells and SMC have shown that differences between arterial-derived and venous-derived endothelial cells may affect SMC behavior [Waybill and Hopkins, 1999]. Our present study was, however, conducted in the absence of endothelial cells, thus indicating that there are inherent differences between VSMC and ASMC in their proliferative response to PDGF. Yang et al. [1998] demonstrated that SMC from failed saphenous vein bypass grafts exhibited enhanced proliferation compared with those from normal internal mammary artery. Recently, Wong et al. [2005] reported that VSMC were more dedifferentiated and demonstrated increased proliferative and synthetic capacity than ASMC in response to FBS. These previous studies and our present results provide a partial basis to explain the higher propensity of graft-venous anastomoses to develop hyperplasia, compared to graft-arterial anastomoses [Cameron et al., 1996; Roy-Chaudhury et al., 2001].

The major findings of this study are that VSMC are more responsive to PDGF stimulation and they are also more susceptible to the tyrosine kinase inhibitor, imatinib, compared to ASMC under PDGF-stimulated culture conditions (Fig. 1). The IC_{50} of imatinib in the VSMC (0.05 μM) was eightfold lower than in ASMC (0.4 μM). These concentrations are lower than or similar to those reported for growth inhibition of cancer cells, mesangial cells and T-lymphocytes (0.1–31.5 μM) [Wang et al., 2000; Boren et al., 2001; Gilbert et al., 2001; Li et al., 2003; Dietz et al., 2004]. Inasmuch as PDGF is prominently expressed in hyperplastic tissues [Roy-Chaudhury et al., 2001] and this growth factor has been implicated in the pathogenesis of the lesion [Nabel et al., 1993], our study provides the theoretical basis for the use of imatinib to inhibit VSMC proliferation and prevent stenosis in venous bypasses and hemodialysis grafts. Further, the differences between VSMC and ASMC should be taken into consideration when considering pharmacological treatments.

To gain mechanistic insights into the differences in susceptibility to imatinib, we also investigated differences in the inhibition of PDGFRβ autophosphorylation by imatinib between the two cell types (Fig. 2). Approximately tenfold higher concentration of imatinib was required for complete inhibition of PDGF-induced autophosphorylation of PDGFR in ASMC, compared to VSMC. This difference paralleled the difference between the two cell types in inhibition of PDGF-induced cell proliferation by imatinib. Thus, these results are consistent with the notion that imatinib's anti-proliferative property in vascular SMC is due to the inhibition of PDGFRβ tyrosine kinase activity and provide further evidence for intrinsic differences in the regulation of PDGFR signaling activities between ASMC and VSMC.

Activation of transmembrane tyrosine kinase growth factor receptors is generally associated with a variety of intracellular signaling events, including the activation of the pro-proliferative PI3K and MAPK signaling pathways. PI3K activates the Akt serine/threonine kinase by generating specific inositol phospholipids which recruit Akt to the cell membrane and enable its activation. Akt mediates cell survival and growth signals by phosphorylating and inactivating pro-apoptotic proteins [Franke et al., 1995]. There is reasonably strong evidence supporting a pivotal role of PI3K/Akt in the proliferation of vascular SMC [Iijima et al., 2002; Adam et al., 2003; Stabile et al., 2003; Liu et al., 2004], although most studies have been performed in aortic SMC from various species. Our present study showed that LY294002, a

specific PI3K inhibitor, reduced PDGF-stimulated ASMC and VSMC proliferation in a dose-dependent manner (Fig. 3), in good agreement with previous studies. At the highest concentrations of LY294002, PDGF-stimulated proliferation was essentially completely abolished in both ASMC and VSMC. These results suggest that the PI3K/Akt pathway is important in mediating both ASMC and VSMC proliferation stimulated by PDGF. In addition, imatinib inhibited PDGF-stimulated Akt phosphorylation at a tenfold lower concentration in VSMC (1 μM) than in ASMC (10 μM; Fig. 4), suggesting that the PI3K/Akt pathway may be partially responsible for the greater sensitivity of VSMC to imatinib compared with ASMC. The concentrations of imatinib required to inhibit Akt phosphorylation in both VSMC and ASMC were, however, approximately tenfold greater than those required to inhibit autophosphorylation of the PDGFβ receptor and cell proliferation, suggesting the possibility of synergistic cross-talk between the PI3K/Akt pathway and other pro-proliferative pathways in SMC, such as the MAPK pathway.

The two best-characterized isoforms of MAPKs, ERK1 (p44MAPK) and ERK2 (p42MAPK), are associated with a number of growth factor receptor pathways, including PDGFRs [Zhan et al., 2003]. Antisense oligonucleotides against ERK1 and ERK2 [Liu et al., 2002] or gene transfer of dominant-negative mutants of ERK [Izumi et al., 2001] have been reported to inhibit SMC proliferation and myointimal hyperplasia thickening in response to arterial injury. In contrast, the ERK-MAPK pathway was dispensable for the PDGF-stimulated proliferation in both bladder SMC and cardiac myofibroblasts [Adam et al., 2003]. In the present study, the MEK1-specific inhibitor, PD98059, which blocks activation of the ERK1/2-MAPK pathway, inhibited proliferation of VSMC and ASMC induced by PDGF in a dose-dependent manner (Fig. 3). At the highest doses of PD98059 employed (50 μM), PDGF-induced proliferation was almost completely abolished in VSMC, but was less than 50% inhibited in ASMC. These data indicate that the ERK1/2-MAPK pathway is important for PDGF-induced VSMC proliferation, but may play a lesser role in ASMC. As in the case of phospho-Akt, imatinib treatment of PDGF-stimulated ASMC and VSMC only modestly reduced the phosphorylation of ERK1/2, at concentrations that

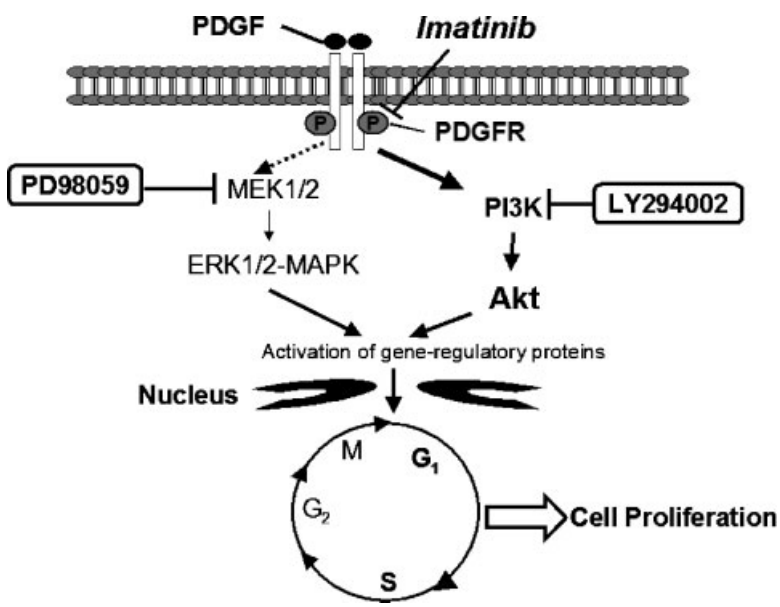

Fig. 7. Proposed intracellular mechanisms underlying the effects of imatinib on PDGF-stimulated SMC proliferation. Upon stimulation with PDGF, there is an increase in the phosphorylation of PDGFR, which is the target of imatinib. As a result of PDGFR blockade by imatinib, Akt phosphorylation and the progression of cell cycle from the G_1 phase to S phase are inhibited. Since imatinib significantly inhibited cell proliferation (Fig. 1B) at concentrations that minimally affected ERK1/2 phosphorylation, interference of the MAPK pathway is unlikely to be the major mechanism by which imatinib inhibits VSMC or ASMC growth. However, synergistic cross talk between the MAPK and PI3K/Akt pathways cannot be ruled out, especially at the level of gene expression regulation.

completely abolished the autophosphorylation of PDGFRβ and cell proliferation (Fig. 5). These results indicate that the ERK1/2-MAPK pathway likely synergizes with other pro-proliferative signaling pathway(s), most likely the PI3K/Akt pathway, in regulating PDGF-dependent cell proliferation, Indeed, synergistic cross-talk between two or more pathways would permit a more precise regulation of a critical process such as growth factor-induced SMC proliferation than could be achieved by a single pathway [Bornfeldt and Krebs, 1999; Campbell et al., 2004]. Preliminary data from our laboratory are consistent with the notion that the ERK1/2-MAPK and the PI3K/Akt pathways might be synergistic in SMC.

In summary, we have demonstrated that VSMC exhibit a greater proliferative response to PDGF than ASMC, and a greater sensitivity to inhibition of PDGF-stimulated proliferation by imatinib. This greater sensitivity of VSMC proliferation to imatinib is reflected in the drug's ability to inhibit VSMC autophosphorylation of PDGFR, as well as the downstream PI3K/Akt and MAPK pathways. A schema of the proposed sequence of intracellular events is depicted in Figure 7. These data highlight important inherent pharmacodynamic differences between SMC from venous and arterial origins that potentially result from different synergistic interactions between signaling pathways. Understanding the molecular and cell-specific signaling processes could be the key to developing effective therapeutic strategies that involve drug combinations targeting various points in one or more signaling pathways. Inasmuch as PDGF is implicated in the pathogenesis of myointimal hyperplasia and that VSMC are more sensitive to the pro-proliferative effects of PDGF, imatinib could well be a useful agent for the inhibition of VSMC and prevention of venous stenosis in hemodialysis grafts and venous bypass grafts. Greater efficacy and tissue selectivity might be achieved by combining imatinib with a drug that targets the PI3K/Akt or MAPK pathways. Such drugs are in various stages of development.

ACKNOWLEDGMENTS

The technical assistance of Craig Kamerath and Ilya Zhuplatov and the suggestions of Dr. John K. Leypoldt, and Dr. Syed F. Mohammad are appreciated.

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