PDGF-DD, a novel mediator of smooth muscle cell phenotypic modulation, is upregulated in endothelial cells exposed to atherosclerosis-prone flow patterns

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Atherosclerosis is a complex disease characterized by the accumulation of lipid and cholesterol deposits within the walls of blood vessels, as well as intimal proliferation and extracellular matrix deposition by phenotypically modulated smooth muscle (SM) cells (SMCs) (1). SMCs within human atherosclerotic lesions and experimental atherosclerosis exhibit a distinct morphological change compared with medial SMCs within the normal vessel wall (32). Along with this morphological change, phenotypically modulated SMCs exhibit decreased expression of a variety of contractile genes, including SMα-actin, SM myosin heavy chain (MHC), SM22α, smoothelin, and h1-calponin (32). Advanced atherosclerotic lesions are characterized by a large lipid and necrotic core covered by a fibrous cap, and the thickness and mechanical properties of these lesions are key determinants of the probability of plaque rupture (3, 12, 13), thrombosis, and subsequent acute myocardial infarction or stroke, the leading causes of death in developed countries (3, 12, 13). Although the precise factors and mechanisms that contribute to plaque rupture are poorly understood, a critical stabilizing factor is believed to be proliferation of and matrix deposition by SMC. In support of this notion, recent evidence suggests that unstable atherosclerotic lesions prone to rupture are characterized by cells expressing lower levels of SMα-actin, indicating a lack of SMCs and/or the preponderance of phenotypically modulated SMCs within these types of lesions (10, 22, 39). Thus an understanding of the factors and mechanisms that control the phenotypic state of SMCs within atherosclerotic lesions is of critical importance.

Platelet-derived growth factor (PDGF)-BB is a secreted molecule known to decrease SMC gene expression (5, 8, 19, 42). PDGF-BB is a homodimer of two B subunits, which can bind to the PDGFαα-, αβ-, or ββ-receptor (37). After binding to the receptor and subsequent receptor dimerization, the receptor is autophosphorylated through intrinsic tyrosine kinase activity, which, in turn, propagates downstream signaling events, including phosphorylation of ERK and Elk-1 and induction of the potent SMC differentiation repressor gene Kruppel-like factor-4 (KLF-4) and transcription factor Sp1 (21, 26, 40, 42). PDGF-DD is secreted as a latent disulfide-linked homodimer, which requires proteolytic cleavage between an NH2-terminal CUB domain and a COOH-terminal PDGF/VEGF homology domain (residues 258–370, also known as the core domain) for full activity (4, 11). Previous studies have demonstrated that PDGF-DD can be activated by urokinase-plasminogen activator in vitro (38).

Fluid shear stress is an important determinant of atherosclerotic lesion development (6, 43). Atherosclerotic lesions characteristically develop at branch points of arteries and bifurca-
tions, where blood flow is complex and highly oscillatory and maintains a low time-average magnitude of shear stress (43). Although the mechanism is unclear, atherosclerosis-prone (atheroprobe) shear stress is believed to be transduced into chemical mediators released by endothelial cells (ECs), which, in turn, influences the recruitment of other cell types, including SMCs, ultimately leading to the formation of atherosclerotic lesions (7). The importance of PDGF ββ-receptor signaling and PDGF-BB in this process has been documented, yet the role of PDGF-DD is virtually unexplored (33).

Here, we demonstrate that PDGF-DD, a PDGF ββ-receptor agonist, decreased the expression of multiple SMC genes, including SM α-actin, SM MHC, SM22α, and h1-calponin, and upregulated the expression of KLF-4, an effector of PDGF-BB-induced SMC marker gene suppression. Of particular importance, we demonstrate that ECs exposed to atheroprobe flow patterns, as occur in vascular regions prone to the development of atherosclerosis, upregulated PDGF-DD expression, thus providing a potential mechanism for SMC phenotypic modulation in diseases associated with altered hemodynamic states.

MATERIALS AND METHODS

Reagents. Full-length and mature cleaved forms of recombinant human PDGF-DD were obtained from ZymoGenetics. U-0126 MEK1/2 inhibitor was purchased from Cell Signaling Technology (Danvers, MA). Plasmid DNA was prepared using Qiafilter MaxiPrep kits (Qiagen).

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Cell culture, PDGF-DD treatment, and transient transfection. Cultured rat aortic SMCs were grown to subconfluence, transiently transfected in insulin-free serum-free medium (IFSM), and stimulated with PDGF-DD or the vehicle as previously described for PDGF-BB (8). These cells and the associated conditions are used routinely by our laboratory and consistently express all the SMC differentiation markers that have been identified.

Real-time RT-PCR. Rat aortic SMCs or human coronary SMCs were treated with PDGF-DD as previously described for PDGF-BB (42). Briefly, subconfluent rat aortic SMCs were treated with IFSFM for 24 h to allow growth arrest and then with PDGF-DD for 24 h in IFSFM at 10 or 30 ng/ml. RNA was harvested, reverse transcribed, and quantified by real-time PCR as previously described (42). Each sample was quantified in duplicate, and each experiment was repeated a minimum of three times, with n = 3.

Mouse SMC harvest from floxed PDGF β-receptor mice and adenoviral injection. Mouse SMCs were obtained from the thoracic aorta of four C57/B16 littermates by manual dissection under a Zeiss dissection microscope. They were plated and amplified in culture for six passages in DMEM with 20% FBS and then switched to DMEM with 10% serum for two further passages. The line was then divided into two parts: the first was infected with an adenovirus expressing Cre recombinase under the control of the cytomegalovirus (CMV) promoter at a multiplicity of infection of 75 for three passages, ~5 days after injection; the second was infected in parallel with a control adenovirus with an empty cassette under the control of the CMV promoter. All animal use protocols were approved by The University of Virginia Institutional Animal Care and Use Committee.

Cells were plated in 10% serum at a density of 1 × 10^4 cells/cm^2 and allowed to grow to ~75% confluence (~24 h). Cells were then washed with Dulbecco’s PBS and allowed to grow arrest in serum-free medium for 24 h. Cells were treated with vehicle, 50 ng/ml human PDGF-AA (Millipore), 50 ng/ml PDGF-BB (Millipore), or 30 ng/ml PDGF-DD (ZymoGenetics) for another 24 h before RNA was harvested, reverse transcribed, and quantified by real-time PCR as previously described (18). Each experiment was repeated a minimum of three times, with n = 3.
harvested in TRIzol reagent (Invitrogen). The iScript cDNA synthesis kit (Bio-Rad) was used to synthesize 0.5 μg of cDNA from each sample. Gene expression for SMα-actin and SM MHC was determined by Bio-Rad quantitative PCR and normalized to expression values of 18S RNA.

Quantitative chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (17). Antibodies included serum response factor (SRF; Santa Cruz Biotechnology, Santa Cruz, CA), Elk-1 (Santa Cruz Biotechnology), acetylated histone H3 (acetyl-H3; Upstate), and acetylated histone H4 (acetyl-H4; Upstate). After immunoprecipitation, 1 ng of DNA from each treatment group was subjected to real-time PCR quantification.

EC-SMC coculture and flow apparatus. The human EC-SMC coculture model has been described previously (15; also see supplemental information in the online version of this article). RNA was harvested and processed for real-time PCR (see above).

In vivo quantification of PDGF-DD expression. Thirty-nine-week-old wild-type C57/Bl6 mice and age-matched apolipoprotein E-deficient (ApoE−/−) mice were euthanized, and aortas were perfused with 4% paraformaldehyde and dissected free. Tissue was embedded in paraffin, and 5-μm aortic sections were collected. Tissue was stained with antibodies to PDGF-D (Santa Cruz Biotechnology) and MAC 2 (Accurate Chemicals, Westbury, NY). Coverslips were applied using Vectashield Hard Set mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Images were acquired using a confocal microscope (model LSM 510-UV, Zeiss) and viewed using the Zeiss LSM 5 Image Browser. Cell counts were performed blindly on aortic arch and abdominal aorta sections from wild-type and ApoE−/− mice (n = 4). The ratio of PDGF-D-positive to DAPI-positive cells was calculated, and Student’s t-test was performed to determine significance at the 0.05 level. Ratios >1 are due to sectioning artifacts, in which cell cytoplasms were present without their corresponding nuclei.

Statistical analysis. Statistical significance was determined by using Student’s t-test. P < 0.05 was considered significant.

RESULTS

PDGF-DD decreased expression of multiple SMC genes. PDGF-BB is a well-known modulator of SMC phenotype and is an agonist at multiple receptors, including PDGFαα-, αβ-, and ββ-receptors (37). PDGF-DD, a recently identified member of the PDGF family, also signals through the PDGF
ββ-receptor (25, 34). Our initial aim was to determine whether PDGF-DD contains SMC phenotypic modulatory activity and whether cleavage is required for this activity. We treated rat aortic SMCs with increasing doses of the uncleaved form, as well as the mature cleaved form, of PDGF-DD. At 60 ng/ml, cleaved PDGF-DD decreased transcript levels of SMα-actin, SM MHC, h1-calponin, SM22α, and the potent SMC-selective transcription factor myocardin by 45%, 88%, 74%, 52%, and 50%, respectively (Fig. 1). The effects on SMα-actin and SM MHC expression were recapitulated in human coronary SMCs (see supplemental Fig. 1). Cleaved PDGF-DD also increased KLF-4 by 251% (Fig. 1). Interestingly, full-length PDGF-DD failed to significantly downregulate SMα-actin, SM MHC, h1-calponin, SM22α, and myocardin. The full-length ligand at 60 ng/ml induced KLF-4, but the response was greatly blunted compared with cleaved PDGF-DD (Fig. 1). These data demonstrate that the cleaved form of PDGF-DD is a selective and potent mediator of SMC phenotypic modulation.

Cleaved form of PDGF-DD inhibited expression of multiple SMC gene promoter-reporters in a dose-dependent manner. PDGF-BB decreases SMC gene mRNA levels and represses the activity of SMC gene promoter-reporter constructs (8, 42). To determine whether PDGF-DD inhibits SMC gene transcription, rat aortic SMCs were transfected with SMC gene promoter-reporter constructs and then with increasing doses of PDGF-DD. At 60 ng/ml, cleaved PDGF-DD resulted in a 72%, 81%, and 65% decrease (P < 0.05) in promoter activity of SMα-actin, SM MHC, and myocardin, respectively (Fig. 2). Furthermore, PDGF-DD at 3 ng/ml increased c-fos promoter activity 57%, and PDGF-DD at 60 ng/ml increased aortic carboxypeptidase-like protein promoter activity 401%. These results indicate that PDGF-DD-induced SMC phenotypic modulation is likely controlled, at least in part, at the level of transcription.

PDGF-DD treatment decreased SMα-actin, SM MHC, and SM22α protein levels in cultured rat aortic SMCs. To extend our analyses of the effects of PDGF-DD on SMC gene expression, we next quantified SMC differentiation marker proteins SMα-actin, SM1 MHC, and SM22α in rat aortic SMCs treated with the cleaved form of PDGF-DD. Importantly, cells showed diminished levels of SMα-actin, SM1 MHC, and SM22α protein after 24, 48, and 72 h of PDGF-DD treatment compared with vehicle control (Fig. 3).

A small interfering RNA to KLF-4 partially blocked PDGF-DD-induced SMC gene repression. We previously showed that KLF-4 can repress the expression of multiple SMC genes, including SMα-actin, SM MHC, and myocardin (26, 27). To define the kinetics of KLF-4 induction by PDGF-DD and the role of KLF-4 in PDGF-DD-induced SMC gene repression, we treated rat aortic SMCs with cleaved PDGF-DD alone or in combination with a small interfering RNA against KLF-4 (siKLF-4). Cell lysates were harvested at the indicated time points and analyzed by Western blot. After 24 and 48 h of treatment with PDGF-DD, levels of KLF-4 protein were significantly increased (Fig. 4A). The siKLF-4 significantly attenuated PDGF-DD-induced decreases in SMα-actin and SM22α protein levels, with little or no effect on changes in SM1 MHC (Fig. 4, B and C). Thus, KLF-4 appears necessary for PDGF-DD-induced repression of SMα-actin and SM22α, whereas alternative KLF-4-independent mechanisms mediate PDGF-DD-induced suppression of SM1 MHC.

An inhibitor of ERK phosphorylation partially blocked PDGF-DD-induced SMC gene repression. ERK is critical in mediating the effects of PDGF ββ-receptor signaling on progression through the cell cycle (29). Phosphorylation of ERK is also required for PDGF-BB-induced SMC gene repression (41). To determine whether ERK phosphorylation is involved in PDGF-DD-mediated SMC gene repression, SMCs were simultaneously treated with 0, 10, or 30 ng/ml PDGF-DD and 10 μM U-0126, an inhibitor of MEK kinase, an upstream activator of
ERK1/2. Cell lysates were harvested at different time points to assess ERK1/2 phosphorylation and SMC protein levels. Consistent with previous reports of the effects of PDGF-BB, the cleaved form of PDGF-DD induced strong and persistent phosphorylation of ERK1 and ERK2 (Fig. 5A). Moreover, the PDGF-DD-mediated decrease in SMα-actin and SM22α protein was strongly attenuated by pretreatment with U-0126 (Fig. 5C). In addition, compared with vehicle-treated cells, SM MHC downregulation by 10 ng/ml PDGF-DD was also partially blocked by treatment with U-0126 (Fig. 5C). At 30 ng/ml PDGF-DD, downregulation of SM MHC remained significant. Interestingly, KLF-4 protein levels appeared unaffected in cells treated with the inhibitor (see supplemental Fig. 2). These results demonstrate that PDGF-DD-induced repression of SMC genes is at least partially dependent on ERK1/2 phosphorylation.

PDGF-DD is a novel SMC phenotypic modulator. PDGF-DD decreased histone marks associated with SMC gene activation and enhanced phosphorylated Elk enrichment at the SMα-actin CARG box. Previous studies from our laboratory (28, 42) and from others (41) demonstrated that

ERK1/2. Cell lysates were harvested at different time points to assess ERK1/2 phosphorylation and SMC protein levels. Consistent with previous reports of the effects of PDGF-BB, the cleaved form of PDGF-DD induced strong and persistent phosphorylation of ERK1 and ERK2 (Fig. 5A). Moreover, the PDGF-DD-mediated decrease in SMα-actin and SM22α protein was strongly attenuated by pretreatment with U-0126 (Fig. 5C). In addition, compared with vehicle-treated cells, SM MHC downregulation by 10 ng/ml PDGF-DD was also partially blocked by treatment with U-0126 (Fig. 5C). At 30 ng/ml PDGF-DD, downregulation of SM MHC remained significant. Interestingly, KLF-4 protein levels appeared unaffected in cells treated with the inhibitor (see supplemental Fig. 2). These results demonstrate that PDGF-DD-induced repression of SMC genes is at least partially dependent on ERK1/2 phosphorylation.

PDGFβ-receptor is necessary for PDGF-DD-induced SMC gene repression. PDGF-DD is known to be an agonist of the PDGF β-receptor (4). To determine whether the PDGF β-receptor is necessary for PDGF-DD-induced SMC gene repression, mouse aortic SMC cultures were derived from mice containing lox p sites flanking exon 2 of both alleles of the PDGF β-receptor gene. Treatment of these cells in culture with adenovirus-expressing Cre resulted in >90% loss of PDGF β-receptor protein (see supplemental Fig. 3). These cells were then treated with PDGF-AA, -BB, and -DD. The results are shown in Fig. 6. In cells treated with PDGF-DD, SMα-actin and SM MHC were decreased by 53% and 57%, respectively. Importantly, in mouse SMCs lacking a functional PDGF β-receptor, PDGF-DD-induced SMC gene repression was completely blocked (Fig. 6). Furthermore, PDGF-BB-induced SMC gene repression was also attenuated by the loss of a functional PDGF β-receptor. These results demonstrate that PDGF β-receptor is necessary for PDGF-BB- and -DD-induced SMC gene repression.
epigenetic modifications contribute to PDGF-BB-induced suppression of SMC marker genes. To determine whether PDGF-DD treatment alone is sufficient to induce epigenetic modifications, we performed quantitative ChIP experiments to measure enrichment of acetyl-H3, acetyl-H4, SRF, and Elk-1 at the SMα-actin CArG box in rat aortic SMCs treated with PDGF-BB or PDGF-DD. After treatment for 0.5 h with either ligand at 30 ng/ml, Elk-1 was significantly enriched and acetyl-H3 and acetyl-H4 in the CArG box-containing region of the SMα-actin promoter were reduced (Fig. 7A). SRF enrichment showed no change at this time point. In contrast, after 24 h of treatment, Elk-1 enrichment returned to vehicle-treated levels in PDGF-DD-treated cells, but cells showed markedly decreased enrichment of SRF, as well as acetyl-H3 and acetyl-H4 (Fig. 7B). Taken together, these results are consistent with a model wherein PDGF-DD-induced repression of SMC marker genes is mediated at early time points by rapid and sustained reduction in histone H3/H4 acetylation and by ERK-dependent phosphorylation of Elk-1, as well as by inhibition of SRF binding to chromatin via epigenetic controls at later time points.

PDGF-DD expression was elevated in intimal lesions within the aortic arch region of ApoE−/− atherosclerotic mice. PDGF-DD profoundly inhibited the expression of multiple SMC genes and induced SMC phenotypic modulation. Given the preponderance of phenotypically modulated SMCs in atherosclerotic lesions, we next wished to determine the expression of PDGF-DD in lesions in an experimental model of atherosclerosis in mice. Aortic arch and descending thoracic aorta sections from ApoE−/− mice fed a Western diet for 20
vascular tree, a region prone to atherosclerotic lesion development (“atheroprone”) (14).

Human ECs exposed to atheroprotective flow patterns showed unchanged levels of PDGF-DD mRNA by real-time PCR analysis compared with cultured ECs exposed to static, or no-flow, conditions (data not shown). Importantly, PDGF-DD mRNA expression was significantly increased by 91% in human ECs exposed to atheroprone flow patterns compared with human ECs exposed to atheroprotective flow patterns (Fig. 9A). In contrast, human SMCs showed no significant change with exposure to altered flow patterns (Fig. 9A).

*PDGF-DD is upregulated in the proximal aorta in vivo.* ECs exposed to atheroprone flow upregulated PDGF-DD expression compared with cells exposed to atheroprotective flow. This suggests that hemodynamic forces play a role in regulating PDGF-DD expression in vivo. To determine whether PDGF-DD was increased in regions of atheroprone flow in vivo, we quantified PDGF-DD by immunofluorescence in the aortic arch (atheroprone region) and abdominal aorta (atheroprotec-

Fig. 6. SMCs lacking PDGF β-receptor failed to repress SMC genes with PDGF-DD or PDGF-BB treatment. Mouse SMCs were obtained from thoracic aorta of C57Bl/6 PDGF β-receptor floxed mice by manual dissection and infected with an adenovirus expressing Cre recombinase (Cre) or an empty cassette, both under the control of the cytomegalovirus (CMV) promoter. Cells were then treated with vehicle, 50 ng/ml human PDGF-AA, 50 ng/ml PDGF-BB, or 30 ng/ml PDGF-DD for 24 h, and RNA was harvested and quantified by real-time PCR. In SMCs possessing a functional PDGF β-receptor, PDGF-DD substantially repressed transcription of SM α-actin and SM MHC 51% and 57%, respectively. In cells lacking PDGF β-receptor, PDGF-DD- and -BB-induced SMC gene repression was abrogated. \(*P < 0.05\).

Fig. 7. PDGF-DD induced early Elk-1 enrichment and late loss of SRF binding and histone acetylation at the SM α-actin promoter within intact chromatin. Subconfluent growth-arrested rat aortic SMCs were treated with PDGF-BB or -DD (30 ng/ml). After 30 min or 24 h of treatment, chromatin was isolated and subjected to quantitative chromatin immunoprecipitation (ChIP) analysis. A: after 30 min of PDGF-BB or -DD treatment, Elk-1 showed 2.5- and 4.5-fold enrichment, respectively, at the SM α-actin promoter. Additionally, there was a trend toward loss of acetylation of histone 3 and 4, with no change in serum response factor (SRF) enrichment. B: PDGF-BB and -DD induced a loss of SRF from the SM α-actin promoter after 24 h of treatment coincident with a loss of acetylation of histone 3 and 4. In contrast, there was no change in Elk-1 enrichment for PDGF-BB and -DD-treated cells at 24 h after treatment. \(*P < 0.05; **P < 0.001.\)
tive region) of wild-type and aged-matched ApoE\(^{-/-}\) mice. Briefly, cross sections from corresponding portions of the aorta were stained with antibodies to PDGF-DD and DAPI and then blindly counted, and the ratio of PDGF-DD-positive to DAPI-positive cells was calculated (Fig. 9B). Interestingly, cells staining positive for PDGF-DD were present in greater abundance in the aortic root than in the abdominal aorta in wild-type mice (Fig. 9B). Importantly, this phenomenon was also observed in age-matched ApoE\(^{-/-}\) mice (Fig. 9B). These results suggest that atheroprone hemodynamic forces may also activate PDGF-DD expression in vivo.

**DISCUSSION**

The purpose of this study was to explore the role of PDGF-DD, a recently identified PDGF \(\beta\)-receptor ligand, in SMC phenotypic modulation. Several novel results are presented. 1) PDGF-DD has the capacity to potently downregulate SMC
than in the abdominal aorta in wild-type and ApoE knockout mice. A greater proportion of cells expressed PDGF-DD in the aortic arch and abdominal aorta sections from wild-type (WT) and age-matched ApoE knockout mice. *P < 0.05.

Fig. 9. PDGF-DD is upregulated in human endothelial cells (ECs) exposed to carotid sinus flow patterns and in the murine aortic root in vivo. A: ECs and SMCs were plated on opposite sides of gelatin-coated porous polycarbonate membrane and grown together in reduced-serum medium. After 24 h of incubation, one of two separate and distinct waveforms, atheroprone or atheroprotective, was applied to the coculture system for an additional 24 h. Real-time PCR showed significantly upregulated expression of PDGF-D in ECs exposed to atheroprone flow patterns compared with ECs exposed to atheroprotective patterns. SMCs showed no change in PDGF-D expression with application of altered flow patterns. B: aortic arch and abdominal aorta sections from wild-type (WT) and age-matched ApoE−/− mice were stained with antibodies to PDGF-DD and with 4,6-diamidino-2-phenylindole (DAPI) nuclear stain. Cells were blindly counted for either signal and are presented as a ratio. A greater proportion of cells expressed PDGF-DD in the aortic arch than in the abdominal aorta in wild-type and ApoE−/− mice. *P < 0.05.

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genes. Moreover, we found that PDGF-DD decreased the activity of several SMC gene promoter-reporter constructs, indicating that changes are mediated, at least in part, at the transcriptional level. 2) PDGF-DD induced ERK phosphorylation and increased KLF-4 expression, two downstream mediators shown here to be required for PDGF-DD-induced SMC gene repression. 3) The PDGF β-receptor was necessary for PDGF-DD-induced SMC gene repression. 4) ChIP studies demonstrate that PDGF-DD decreased binding of SRF to CArG cis-elements within the endogenous SM α-actin promoter in intact chromatin. In addition, there was early recruitment of Elk-1 to the promoter, as well as a later decrease in histone H3 and H4 acetylation. 5) PDGF-DD was increased in the neointima of atherosclerotic lesions from the aortic arch of ApoE−/− atherosclerotic mice. 6) In a coculture model of ECs and SMCs, human ECs exposed to atheroprone hemodynamic shear stress significantly upregulated the expression of PDGF-DD, while cells expressing PDGF-DD were more abundant in vivo in regions of atheroprone flow. These latter observations are consistent with a model in which ECs, located within atherosclerosis-prone regions of the vasculature, secrete and release PDGF-DD in response to oscillatory and low-shear-stress blood flow patterns. This secretion, in turn, may induce SMCs to alter their phenotype, a cellular change observed in athero-

sclerotic lesions. Importantly, these results establish a potential link between atherosclerosis-prone blood flow patterns, PDGF-DD secretion by ECs, and SMC phenotypic modulation.

The SMC phenotypic modulatory activity of PDGF-BB is well described (5, 8, 19, 41, 42). However, the role of PDGF-DD in this process and the receptor responsible are virtually unexplored. The specific receptor binding profile of PDGF-DD, as well as a unique loss-of-function approach, allowed us to test the dependence of SMC gene repression on PDGF β-receptor signaling. Indeed, the above-described experiments establish for the first time that the PDGF β-receptor is necessary for SMC gene repression induced by PDGF-DD. Moreover, observations that PDGF-BB induced repression of SMC marker genes was also abrogated in PDGF β-receptor-knockout SMC (Fig. 6) are the first to conclusively show that these effects are dependent on the β-receptor, in that previous studies relied solely on use of pharmacological inhibitors that may affect other receptor pathways.

The binding receptor profile of PDGF-DD is controversial. Conflicting reports suggest that PDGF-DD is a specific β-receptor agonist or has activity at the α- and/or α/β-receptor heterodimer (4, 18). Indeed, the ability of PDGF-DD to bind the PDGF α- or α/β-receptor has not been established (2). Thus we cannot rule out the possibility that PDGF-DD acts, at least in part, through the PDGF α-receptor.

Results demonstrating that PDGF-DD and -BB enhanced early Elk-1 enrichment, late loss of SRF enrichment, and histone H3 and H4 acetylation suggest that these chromatin modifications are important for PDGF-induced SMC gene repression. The mechanisms for PDGF-BB-induced SMC gene repression have been explored in depth (26, 28, 40–42). This activity has been shown to be dependent on multiple repressor pathways (for review see Ref. 21), including ERK1/2 phosphorylation (41), Sp1 (40), histone deacetylation-2 (42), and p38 (16). Furthermore, PDGF-BB-induced SMC gene repression correlated with decreased SRF (26, 28, 40, 42) and decreased myocardin enrichment (41), loss of histone H4 acetylation (28, 42), and Elk-1 enrichment (41, 42) at various SMC gene promoters among other mechanisms (21). A similar loss of SRF, decreased histone H3 and H4 acetylation, and Elk-1 enrichment between PDGF-BB and -DD indicate that these epigenetic changes are downstream of PDGF β-receptor binding and do not require PDGF α-receptor agonism.

KLF-4 and phosphorylation of ERK are shown here to be required for PDGF-DD-induced SM α-actin and SM22α repression. ERK1/2 phosphorylation is necessary for PDGF-BB-induced SMC gene repression (41), and KLF-4 is induced by PDGF-BB and capable of repressing myocardin-induced SMC gene activation (26). Here, we demonstrate that KLF-4 and ERK are additive in their ability to mediate PDGF-DD-induced SM α-actin and SM22α gene repression. siKLF-4 showed no effect on ERK1/2 phosphorylation or protein levels. Conversely, MEK1/2 inhibition with two separate compounds showed no effect on KLF-4 expression (see supplemental Fig. 2). Thus these data provide novel evidence that KLF-4 and ERK1/2 are independent downstream mediators of PDGF β-receptor-induced SMC gene repression. However, further studies are needed to determine the signaling pathways responsible for KLF-4 induction and SM MHC repression.

Our findings that PDGF-DD is upregulated in ECs exposed to atheroprone hemodynamic flow patterns suggest a novel
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pathophysiological role for PDGF-DD. Although evidence supports a role for PDGF-BB in the formation of atherosclerotic lesions in regions of disturbed flow (36), surprisingly few studies have tested the role of PDGF-DD, despite the clear importance of PDGF β-receptor signaling (23, 33). In addition, it is interesting to speculate that PDGF-DD may contribute to the formation of intimal masses, which represent accumulations of SMCs in the intima that preferentially develop in regions of disturbed flow in humans at an early age (39). An increased number of PDGF-DD-positive cells in regions prone to the development of atherosclerosis in wild-type mice, which do not develop atherosclerotic lesions spontaneously, is consistent with this hypothesis. Whereas much additional work is needed to test these possibilities, the results of the present studies indicate that further study of the potential role of PDGF-DD in formation of intimal masses in humans is warranted.

The evolution of a distinct PDGF family member became an outstanding question upon the discovery of PDGF-DD. By agonizing a distinct set of receptors in vivo, it is believed that PDGF-DD mediates physiological effects that are different from those of other PDGF family members. Importantly, the receptor binding profile of PDGF-DD remains controversial; thus a discussion of the role of PDGF-DD in vivo is limited. However, a discussion of α- vs. β-receptor roles is important. Although somewhat controversial, results from a number of in vitro studies have provided evidence that PDGF α-receptor signaling selectively mediates DNA and protein synthesis (20, 37), but not migration (9), whereas PDGF β-receptor signaling induces DNA synthesis (20) and migration (9). Because of the receptor binding properties of PDGF-DD, it is interesting to speculate that perhaps PDGF-DD contributes to disease states where there is both migration and proliferation of β-receptor-expressing cells. Examples would include tumor growth and invasion, where mural cells must migrate and invest newly developed tumor capillaries during arterialislation, as well as atherosclerosis, which is characterized by intimal migration and proliferation of SMC. Indeed, evidence has emerged implicating PDGF-DD in tumorigenesis and atherosclerosis (24, 34). In contrast, PDGF-AA or selective PDGF α-receptor activation may primarily mediate SMC growth without migration, as occurs during development of medial hypertrophy in hypertension (for review see Ref. 31). However, there are a number of critical unresolved questions. 1) Does PDGF α- vs. β-receptor signaling mediate fundamentally different SMC responses in vivo as opposed to responses in cultured cells, which have been the focus of virtually all studies comparing these two signaling pathways? 2) What is the cross talk between PDGF α- and β-receptor signaling pathways within SMC in vivo that might alter responses to PDGF-BB vs. PDGF-DD? 3) What is the exact receptor binding profile of PDGF-DD? Indeed, a key question is as follows: Do SMC in vivo, which express both PDGF α- and β-receptors, show differences in gene expression patterns when stimulated with PDGF-BB vs. PDGF-DD? Resolving these questions will require development of complex mouse lines that exhibit conditional and SMC-selective knockout of these receptors in combination with models of vascular injury, atherosclerosis, and hypertension.

In summary, the results of the present studies reveal a novel role for PDGF-DD in SMC physiology and as a potential chemical mediator of hemodynamic shear stress. We demonstrate the conservation of molecular mechanisms between PDGF-BB and -DD, implicating early Elk-1 enrichment and late loss of SRF and histone acetylation as effectors of SMC gene repression. Finally, we demonstrate that ERK signaling, KLF-4, and the PDGF β-receptor are necessary for PDGF-DD-induced SMC gene repression. Importantly, these data establish a link between low/oscillatory shear stress blood flow, such as that which occurs in regions of atherosclerotic lesion formation, EC-mediated PDGF-DD expression, and SMC phenotypic modulation. Future experiments will be aimed at delineating the specific role of PDGF-DD in the progression of atherosclerosis and other diseases associated with disturbed blood flow, as well as the role of PDGF α-receptor signaling in PDGF-DD-induced SMC gene repression.

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