Transforming Growth Factor-\(\beta\)-Dependent Growth Inhibition in Primary Vascular Smooth Muscle Cells Is p38-Dependent

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ABSTRACT

Vascular smooth muscle cells (VSMCs) constitute the major cellular component of the vessel tunica media. VSMC proliferation is a key feature in developing vessels and pathological states such as atherosclerosis and restenosis. Transforming growth factor (TGF)-\(\beta\) is a key regulator of VSMCs, but its effect on VSMC proliferation and apoptosis are controversial. Here, we characterized TGF-\(\beta\) effects on basal-, serum-, and platelet-derived growth factor-BB-induced primary mouse VSMC proliferation. TGF-\(\beta\) led to potent growth inhibition of VSMCs isolated from normal mouse aortae without inducing apoptosis. Growth inhibition by TGF-\(\beta\) was due to G\(_0/G_1\) arrest. Next, we explored distinct signaling pathways activated by TGF-\(\beta\) and the effects of pharmacological inhibition of these. TGF-\(\beta\) led to activation of Smad2/3, p38, p42/44, and c-Jun NH\(_2\)-terminal kinase (JNK) pathways, assessed by phosphorylation, immunofluorescence, and reporter gene analysis. TGF-\(\beta\)-dependent growth inhibition was specifically attenuated by pharmacological blockade of the TGF-\(\beta\) type I receptor (T\(\beta\)RI) kinase or p38 mitogen-activated protein kinase pathways, whereas blockade of p42/44 or JNK kinases did not influence the effect of TGF-\(\beta\) on VSMC proliferation. T\(\beta\)RI kinase inhibition blocked all downstream pathways including Smad and p38 phosphorylation. In contrast, p38 inhibition did not alter Smad function, as assessed by translocation or reporter gene expression, but selectively inhibited p38 activity. These results demonstrate that TGF-\(\beta\) acts as a potent antiproliferative mediator in VSMCs, irrespective of the proliferative stimulus, without inducing apoptotic effects. The anti-proliferative effect of TGF-\(\beta\) is due to G\(_0/G_1\) arrest and mediated primarily by the p38 pathway, suggesting that p38 kinase is central to TGF-\(\beta\)-mediated growth inhibition in primary mouse VSMCs.

Atherosclerosis is a major cause of morbidity and mortality in the Western world. Several risk factors such as high cholesterol levels, cigarette smoking, arterial hypertension, diabetes mellitus, obesity, or genetic dispositions have been described to induce or promote atherogenesis (Dzau et al., 2002; Mallat and Tedgui, 2002; Stocker and Keaney, 2004). Pathophysiologically, atherosclerosis is characterized by distinct arterial intimal lesions leading to plaque formation and subsequent narrowing of the vessel lumen. These pathological changes develop in a chronological order from fatty streak formation to plaque rupture and thrombosis. Main molecular regulators of atherogenesis are oxidative stress, lipid influx, cellular recruitment and activation, and vascular smooth muscle cell (VSMC) migration and proliferation (Dzau et al., 2002). In the early stages of atherosclerotic plaque formation, VSMCs migrate into and accumulate within the arterial intima, whereas in later stages, VSMC proliferation is a main determinant of plaque size and stability (Dzau et al., 2002; Owens et al., 2004).

TGF-\(\beta\) represents a pleiotropic growth factor and key regulator of cell migration, differentiation, proliferation, and apoptosis (Roberts, 1998). TGF-\(\beta\) signaling is initiated upon ligand binding to the TGF type II receptor (T\(\beta\)RII), which then recruits the type I receptor T\(\beta\)RI into the receptor complex. Subsequent phosphorylation of the signaling receptor T\(\beta\)RII by the constitutively active kinase activity of T\(\beta\)RII initiates the phosphorylation and activation of the intracellular mediators Smad2 or Smad3. Phosphorylated Smad2/3...
then undergoes nuclear translocation, which may be due to complex formation with Smad4. Once in the nucleus, Smads interact with several transcriptional activators or repressors and modify gene expression (Eickelberg, 2001; Shi and Masague, 2003; ten Dijke and Hill, 2004). Although Smad-dependent responses represent one of the main signaling systems utilized by TGF-β receptors, alternative signaling components are able to mediate TGF-β-induced biological effects, such as the MAP kinases p38 or JNK (Miyazono et al., 2000; Yue and Mulder, 2001). In this context, the biological response to TGF-β is determined by the coexpression, coactivation, and cross talk of simultaneously activated signaling systems, which can explain cell-type-restricted patterns of biological responses to TGF-β.

The role of TGF-β in atherosclerosis is controversial. Several studies have assigned a protective role for TGF-β in atherosclerosis, whereas others have shown that TGF-β overexpression is associated with disease progression (Mallat and Tedgui, 2002; Grainger, 2004; Owens et al., 2004). In animal models, endothelial overexpression of TGF-β in uninjured arteries leads to reversible formation of a cellular and matrix-rich neointima with transdifferentiation of VSMCs into chondrocytes (Schulick et al., 1998). TGF-β is also described to mediate luminal narrowing and lesion formation in restenosis following angioplasty (Smith et al., 1999; Ryan et al., 2003). In contrast, depletion of TGF-β was sufficient to exacerbate lesion development in ApoE−/− mice (Mallat et al., 2001; Lutgens et al., 2002), indicating a beneficial role of TGF-β in atherogenesis. In vitro, TGF-β exerts antiproliferative effects on various cell types including epithelial and smooth muscle cells (Roberts, 1998). It remains to be elucidated, however, whether TGF-β-induced apoptosis contributes to its growth inhibitory effect and which signaling systems determine the antiproliferative response to TGF-β in VSMCs. In this study, we therefore sought to test our hypothesis that Smad-independent pathways contribute to phenotypic switching of VSMCs. Here, we characterized the proliferative and apoptotic response of primary VSMCs to TGF-β and elucidated the relative contribution of Smad and MAP kinase pathways to the effects elicited by TGF-β.

Materials and Methods

Materials. The following antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA): phospho-p38 MAPK (Thr180/Tyr182, no. 9211), total p38 MAPK (no. 9212), phospho-p42/p44 MAPK (Thr202/Tyr204, no. 5120), total p42/44 MAPK (no. 9102), phospho-JNK (Thr183/Tyr185, no. 9251), total JNK (no. 9252), and phospho-Smad2 (Ser465/467, no. 3101). Antibody against Smad1,2,3 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA; sc-7960), and anti-α smooth muscle actin was from Chemicon International (Temecula, CA; cb1 171). Anti-Smad2/3 was purchased from BD Biosciences (San Jose, CA; no. 610842) and Alexa568-conjugated annexin-V from Roche Diagnostics (Mannheim, Germany; no. 0370126001). Fluorescein isothiocyanate (FITC)-conjugated goat α-mouse IgG was from Zymed Laboratories (South San Francisco, CA; no. 81-6511).

Chemical inhibitors used in this study were as follows: SB203580 and SB202190 from Cell Signaling Technology Inc. (Beverly, MA); PD098059 and U31728 from Calbiochem (San Diego, CA); and SB431542 from R&D Systems (Minneapolis, MN). Expression plasmids for wild-type and constitutive active (ca) ALK5 were kindly provided by Carl-Hendrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden).

Isolation and Culture of VSMCs. Male C57Bl/6 mice were sacrificed, the aorta removed, and VSMCs isolated by the explant method, as previously described (Sedding et al., 2005). VSMCs were cultured in Dulbecco’s modified Eagle’s medium-F12 supplemented with 10% fetal bovine serum (FBS) (In Vitrogen, Carlsbad, CA) and maintained under 5% CO2 at 37°C in a humidified atmosphere. VSMCs exhibited typical spindle-shaped morphology throughout culture and stained positive for smooth muscle-specific α-actin. For all experiments reported in this study, only passages 4 to 8 were used. Quiescence, when indicated, was achieved by serum withdrawal for 24 h.

Proliferation Assays. For cell counts, VSMCs were plated into 24-well culture plates at a density of 15 × 10³/well. The following day, wells were treated with the indicated stimuli. Cells were then harvested by trypsinization and counted using a hemocytometer after the indicated time points. In select experiments, cell counts were confirmed by monitoring DNA synthesis by [3H]-thymidine incorporation assays. For this, cells were seeded into 24-well plates and treated as indicated. Cells were then pulsed with 0.6 μCi of [3H]-thymidine (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 4 h. Plates were washed twice with cold PBS and incubated with ice-cold 10% trichloroacetic acid for 30 min at 4°C. Subsequently, samples were solubilized in 0.5 M NaOH. The contents of each well were then transferred into scintillation fluid and incorporated radioactivity counted in a scintillation counter.

Apoptosis Assay. Cells were grown in T-25 culture flasks to a density of 90% before being treated as indicated. Following trypsinization, cells were centrifuged and resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2) containing Alexa568-conjugated annexin-V and propidium iodide (PI; Roche Diagnostics). After incubation, apoptotic cells were detected by annexin-V binding using FACS Scan according to the manufacturer’s instructions (BD Biosciences, Franklin Lakes, NJ). Necrotic cells were excluded by PI staining.

Western Blot. VSMCs were grown on six-well culture dishes to a density of 90% before the indicated stimulations. Cells were then harvested in lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, and Complete inhibitor (Roche Diagnostics)]. After 15-min incubations on ice, lysates were centrifuged for 20 min and supernatants collected into fresh tubes. Protein content was determined using the Protein DC Assay (Bio-Rad, Hercules, CA) and 20 μg of each sample separated on 10% SDS-polyacrylamide gels. Following electrophoresis, proteins were blotted to polyvinylidene difluoride membranes (GE Osominsis, Inc., Minnetonka, MN). Blocking of membranes was performed using 5% nonfat dry milk in PBS for 1 h, after which incubations with primary antibodies diluted 1:2000 was performed overnight. After incubation with the respective horseradish peroxidase-conjugated secondary antibodies (Pierce Chemical, Rockford, IL), proteins were detected using ECL plus reagents (Amersham Biosciences AB) according to the manufacturer’s instructions.

p38 Kinase Assay. Assays were performed using the p38 MAP Kinase Assay Kit from Cell Signaling Technology Inc. Briefly, 200 μg of cell lysates was immunoprecipitated with immobilized antibody against phospho-Thr180/Tyr182 p38 MAPK. After washing, the pellet was resuspended in kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium vanadate, and 10 mM MgCl2), and kinase reactions were initiated by the addition of 200 μM ATP and 2 μg of ATF-2 fusion protein. After 30 min at 30°C, the reaction was terminated by the addition of 2× SDS sample buffer, and samples were subjected to electrophoresis and blotting. p38 MAPK kinase activity was detected by probing the membrane with antibody against phospho-ATF-2.

Flow Cytometric Cell Cycle Analysis. Cells were harvested by trypsinization, fixed overnight with 75% methanol at −20°C, washed
in PBS, and incubated with 100 μg/ml RNase (Roche Diagnostics) and 10 μg/ml PI in PBS for 1 h at 37°C. Samples were analyzed for DNA content using a high-speed cell sorter (EPICS Altra; Beckman Coulter, Fullerton, CA). Data were computer-analyzed with commercially available software (Multicycle; Phoenix Flow Systems, San Diego, CA).

**Reporter Gene Assay and Transfections.** Assays were analyzed using the Luciferase Assay System (Promega, Madison, WI). VSMCs were plated at a density of 10 × 10^3/well in 48-well plates. The next day, cells were transfected using FuGene (Roche Diagnostics) transfection reagent and 200 ng of reporter construct per well. After 4 h, cells were treated as indicated and incubated overnight. Plates were washed with PBS, lysed in lysis buffer (25 mM Tris, pH 7.8, 2 mM dithiothreitol, 10% glycerol, and 1% Triton X-100), and luminescence was measured according to the manufacturer’s instructions.

**Immunofluorescence.** VSMCs were seeded on eight-well chamber slides at 10 × 10^3 per well. After indicated stimulations, cells were washed with PBS and fixed in ice-cold methanol. Slides were blocked in 5% FBS and then incubated with primary and secondary FITC-labeled antibodies, as depicted. Nuclei were visualized by 4,6-diamidino-2-phenylindole staining (Roche Diagnostics) and individual cells analyzed by deconvolution fluorescence microscopy using the Leica AS-MDW (Leica, Wetzlar, Germany).

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**Results**

**TGF-β1 Inhibits VSMC Proliferation in the Absence of Apoptosis.** Initially, we characterized whether TGF-β1 would elicit an antiproliferative and/or proapoptotic response in primary mouse VSMCs. Figure 1A shows that addition of TGF-β1 significantly attenuated VSMC growth in a time-dependent manner. Although VSMC proliferation increased 5.4-fold under serum stimulation over 72 h (81.1 ± 6.6 × 10^3 versus 15 × 10^3 cells, 5.4-fold increase), the addition of TGF-β1 at 2 ng/ml significantly decreased serum-dependent proliferation over 72 h (29.4 ± 4.5 × 10^3 versus 15 × 10^3 cells, 1.96-fold increase). This antiproliferative effect of TGF-β1 was dose-dependent (Fig. 1B, analyzed after 48 h) and was observed not only in serum-stimulated VSMC proliferation but also in PDGF-BB-stimulated cells (data not shown). The reduction in cell proliferation in response to TGF-β1 was observed both by direct cell counts and thymidine incorporation (Fig. 1, A–C). Next, we asked whether this growth inhibitory effect of TGF-β1 coincided with an induction of apoptosis in mouse primary VSMCs. Figure 1D shows that TGF-β1 did not induce apoptosis in mouse primary VSMCs over a time period of up to 28 h, whereas the positive control

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**Fig. 1.** TGF-β1 inhibits VSMC proliferation in the absence of apoptosis. Mouse aortic VSMC numbers were assessed at the indicated time points with or without TGF-β1 treatment of 2 ng/ml (A; *p < 0.005 versus without TGF-β1) and in response to different concentrations of TGF-β1 after 48 h of treatment (B). C, thymidine incorporation of primary mouse VSMCs. Apoptosis was analyzed at the time points indicated, and percentage of apoptotic cells was determined by annexin V staining with subsequent fluorescence-activated cell sorting analysis (D). B and C, *p < 0.005 versus control; #, p < 0.005 versus 10% FBS. All experiments were performed in quadruplicate, with each experiment being performed with at least two different isolations of VSMCs.
staurosporine elicited a significant increase in the percentage of apoptotic cells. TGF-β1 did not induce apoptosis over a concentration range from 0.1 to 10 ng/ml (data not shown).

**TGF-β1 Induces Phosphorylation of Smad and MAP Kinase Pathways.** To dissect the contribution of different signal transduction pathways to TGF-β1-induced growth inhibition, we analyzed Smad and MAPK phosphorylation kinetics in response to TGF-β1 stimulation over a time frame of 24 h (Fig. 2A). Rapid phosphorylation of Smad2 was detectable as early as 15 min after stimulation of VSMCs with 2 ng/ml TGF-β1. Smad2 phosphorylation peaked until 1 h, after which it gradually decreased, but was still detectable by 24 h. TGF-β1 also induced phosphorylation of p38 MAP kinase in primary VSMCs. Phosphorylated p38 was detectable after 1 h and returned to baseline levels after 8 h. Phosphorylation of p42/44 showed an early and transient signal after 15 min, with a second peak of phosphorylation at 4 h. JNK kinase showed a similar pattern of phosphorylation as p42/44, with an early signal at 15 min, and a second signal after 4 h. To demonstrate that phosphorylation of Smad2 and p38 was directly due to the kinase activity of TβRI/ALK5 and not to coactivated pathways, we next transfected primary VSMCs with a constitutively active ALK5 expression construct, which mimics kinase activity in the absence of ligand treatment. As shown in Fig. 2B, transfection of VSMCs with caALK5 led to a significant phosphorylation of Smad2 and p38 in a time-dependent manner, indicating that ALK5 kinase activity is sufficient for Smad2 and p38 phosphorylation.

**Inhibition of TβRI and p38 Kinase Activity Attenuates Growth Inhibition by TGF-β1.** After determination of the phosphorylation kinetics shown in Fig. 2, we next sought to analyze whether interference with these pathways would affect TGF-β1-dependent growth inhibition in VSMCs. To characterize this, we used specific inhibitors of the TβRI, p38, p42/44, and JNK kinases (SB431542, SB203580, U0126, and JNK inhibitor II, respectively) and evaluated their effect on serum-induced VSMC proliferation. As depicted in Fig. 3, only inhibition of the p42/44 pathway led to a significant reduction in baseline VSMC proliferation in the presence of 10% serum. In contrast, inhibition of TβRI and p38 activity by SB431542 and SB203580, respectively, led to a complete loss of the growth inhibitory effect of TGF-β1 (Fig. 3), whereas neither inhibitor affected baseline proliferation of VSMCs in the presence of serum. Both of these inhibitory effects were concentration-dependent and detected over a

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**Fig. 2.** TGF-β1 induces rapid phosphorylation of Smad and MAP kinase pathways. VSMCs were treated for the indicated time points with TGF-β1 (2 ng/ml), and total cell lysates were prepared. Equal amounts of proteins (20 μg) were separated by SDS-polyacrylamide gel electrophoresis, and the activity and expression of the indicated pathways analyzed by Western blotting (A). Phosphoproteins were detected via phospho-specific antibodies as indicated. Equal loading was confirmed via pan-specific antibodies and an antibody against smooth muscle actin. In B, VSMCs were transfected with an expression plasmid of caALK5 or the corresponding empty vector, and phosphorylation of Smad2 and p38 was analyzed 24 and 48 h after transfection.
concentration range of 0.1 to 10 μM (data not shown). Interfering with the JNK pathway by addition of JNK inhibitor II had no effect on baseline or TGF-β-dependent cell numbers.

We next sought to elucidate whether the observed changes in cell numbers were also evident upon analyzing cell cycle entry in response to serum TGF-β. Synchronized VSMC populations exhibited an 81% G0/G1, 2.2% S, and 16.8% G2/M phase distribution (Fig. 4, top left graph). Induction of proliferation by serum stimulation resulted in a significant increase in the G2/M to G0/G1 ratio, with an accompanying increase in S phase populations (Fig. 4, left column). This cell cycle entry was inhibited by TGF-β, which led to a G0/G1 arrest observed at 18 and 24 h (Fig. 4, second column). Pretreatment of VSMCs with SB431542 or SB203580 resulted in attenuation of this inhibitory effect of TGF-β (Fig. 4, third and fourth columns, respectively), which was similar to the effect observed on cell proliferation (compare Fig. 4 with Fig. 3). Inhibition of the TβRI or p38 pathways allowed VSMCs to enter and complete cell cycle progression even in the presence of TGF-β, as evidenced by similar G2/M populations at 24 h (Fig. 4, bottom row).

Inhibition of p38 Activity Does Not Alter Smad Activity. Our data thus far demonstrated that inhibition of the TβRI kinase, but also p38 inhibition, completely blocked TGF-β-induced growth inhibition in VSMCs. This raises the question of whether the kinase inhibitors used in this study are specific for their targets (ALK5, p38, and p42/44 kinase, respectively) or whether they may also inhibit one or more of the other pathways investigated. To clarify this issue, we investigated phosphorylation kinetics of Smad2,3, p38, and p42/44 in response to TGF-β after preincubating cells with SB431542, SB203580, or U0126, respectively, as depicted in Fig. 5A. As expected, the TβRI kinase (SB431542) and the p42/44 (U0126) inhibitor completely abolished Smad2 and p42/44 phosphorylation, respectively. Neither inhibitor affected any of the other pathways, indicating a high specificity.

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Discussion

TGF-β is a highly pleiotropic cytokine capable of inducing a variety of biological effects that ultimately control the balance between cellular differentiation and proliferation (Shi and Massague, 2003). Many of the genomic effects of TGF-β can be attributed to activation of Smad2/3 molecules, sequence-specific transcription factors directly activated by the TGF-β type I receptor (TβRI), and their subsequent interaction with DNA. Although Smad activation is regarded as a universal response of all cell types in response to TGF-β, it has recently become clear that several Smad-independent
pathways are coactivated in a cell-specific manner in response to TGF-β (Roberts, 1998; Miyazono et al., 2000; Eckelberg, 2001). Thus, the biological response to TGF-β depends on the coordinated interplay of different signaling pathways. The ability to selectively manipulate a biological response to TGF-β will therefore rely on our understanding of this interplay and the relative contribution of a specific pathway to complex cellular outcomes such as proliferation or apoptosis.

In the current study, we extensively analyzed TGF-β-induced Smad and MAP kinase pathways in primary VSMCs, a cell type integrally involved in vascular remodeling during development and diseases such as atherosclerosis or restenosis. We could demonstrate that TGF-β elicits a potent growth inhibitory effect in VSMCs, which was observed in serum- and PDGF-BB-stimulated cells. This antiproliferative effect was due to induction of G0/G1 arrest in TGF-β-treated cells, irrespective of the proliferative stimulus. This biological response coincided with rapid and prolonged phosphorylation and activation of Smad2/3, p38, and JNK kinases. Treatment of VSMCs with TGF-β also stimulated transient phosphorylation of p42/44. Pharmacological inhibition of the kinase activity of TβRI (using SB431542) completely blocked downstream phosphorylation events, including Smad and p38 phosphorylation, which led to an attenuation of TGF-β-dependent growth inhibition in VSMCs. Accordingly, TGF-β-induced p38, p42/44, and JNK phosphorylation originates from the receptor complex, either directly, as has been demonstrated for p38 kinase activity (Yu et al., 2002), or indirectly, via Smads or other potential interaction partners.

More specifically, pharmacological inhibition of the p38 pathway (using SB203580; English and Cobb, 2002) resulted in complete attenuation of TGF-β-dependent growth inhibition, but this occurred in the absence of any inhibitory effect on Smad signaling, as analyzed by phosphorylation, nuclear translocation, or reporter gene expression (Fig. 5). Moreover, p38 inhibition had no effect on baseline, serum-, or PDGF-BB-stimulated proliferation of VSMCs, indicating that p38 specifically mediates the TGF-β-induced changes, leading to cell cycle arrest and subsequent growth inhibition. Thus, we conclude that p38 kinase is the main mediator of growth inhibition induced by TGF-β in VSMCs. In contrast, p42/44 activity was required for proliferation of VSMCs because inhibition of p42/44 phosphorylation significantly down-regulated serum- or PDGF-BB-stimulated proliferation (Fig. 3), as suggested before (Kavurma and Khachigian, 2003; Zhan et al., 2003), whereas it had no effect on the TGF-β response. Earlier investigations have suggested that p38 activity is also implicated in serum-induced proliferation of VSMCs by
using Ki67 as a marker and thymidine incorporation assays (Zhao et al., 2002). Recently, p38 activity has also been implicated as an essential mediator of TGF-β-stimulated vascular endothelial growth factor synthesis in aortic smooth muscle cells (Yamamoto et al., 2001), suggesting a general role for p38 in mediating TGF-β-induced responses of this

Fig. 5. Inhibition of p38 activity does not alter Smad activity. A, VSMCs were cultured in growth medium (10% FBS) and treated for the indicated time points with TGF-β1 (2 ng/ml) in the presence or absence of SB431542, SB203580, or U0126, as indicated, and total cell lysates prepared. Equal amounts of proteins (20 μg) were separated by SDS-polyacrylamide gel electrophoresis, and Western blot analyses were performed. Phosphoproteins were detected via phospho-specific antibodies as indicated. Equal loading was confirmed via pan-specific antibodies and an antibody against smooth muscle actin. B, p38 kinase activity 60 min after TGF-β1 stimulation, in the presence or absence of SB431542 or SB203580, as indicated, was assessed by immunoprecipitation of active p38 and phosphorylation of ATF-2. C, Smad-dependent reporter gene activity (pCAGA12, a kind gift of Peter ten Dijke, Netherlands Cancer Institute, Amsterdam, The Netherlands) was analyzed in primary VSMCs in response to TGF-β1 in the presence or absence of SB431542 or SB203580, as indicated. Values given are represented as -fold induction to unstimulated controls, performed in quadruplicate (*, p < 0.005 versus the corresponding untreated controls). D, TGF-β1-dependent nuclear translocation of Smad2 is p38-independent. VSMCs were plated on glass slides, treated as indicated, and Smad2/3 localization assessed by immunofluorescence analysis. Smad2/3 was detected by FITC-labeled secondary antibody, and nuclei were visualized by 4,6-diamidino-2-phenylindole staining.
cell type. Although many studies to date have assigned a proliferative role for p38 kinase (Zhao et al., 2002; Kavurma and Khachigian, 2003; Zhan et al., 2003), a recent study by Kamaraju and Roberts (2005) demonstrated that the antiproliferative responses to TGF-β in a breast cancer epithelial cell line required p38. This indicates that the biological effect of p38 activity is highly context- and stimulus-specific and is ultimately determined by extensive cross talk with other kinase systems such as the TGF-β receptors.

Does the observed growth inhibition induced by TGF-β represent a pathophysiologically relevant effect? Phenotypic switching of VSMCs is characterized by profound yet reversible changes in cellular differentiation and proliferation (Owens et al., 2004). This process is believed to play a key role in atherosclerosis, where the role of the VSMC appears to vary depending on the stage of disease progress, albeit other cell types such as macrophages, neutrophils, endothelial cells, or circulating stem cells also play essential roles (Sata et al., 2002; Caplice et al., 2003; Owens et al., 2004; Stocker and Keaney, 2004). VSMCs seem to play maladaptive roles in early atherosclerotic lesion development and progression, but they likely play beneficial roles in advanced disease by stabilizing fibrous plaques (Owens et al., 2004). In this respect, TGF-β-dependent signaling in VSMCs would elicit beneficial effects at both stages of this disease. Preventing VSMC proliferation could diminish lesion formation in early disease, whereas growth inhibition in the absence of apoptosis would still stabilize fibrous caps in advanced disease. This has been elegantly suggested in ApoE−/− mice, where neutralizing antibodies against TGF-β1, -β2, or β3 accelerated atherosclerotic lesion development with increased inflammatory cells and decreased collagen content (Mallat et al., 2001; Lutgens et al., 2002), indicating a protective role for TGF-β in advanced disease. In the same genetic model, abrogation of TGF-β signaling in T cells also accelerates atherosclerosis, causing unstable lesions with reduced collagen and increased inflammation (Robertson et al., 2003). On the other hand, overexpression of TGF-β in endothelial cells causes neointima formation with enhanced matrix deposition (Schulick et al., 1998), emphasizing the complexity of this system in vivo and the need to fully understand specific signaling systems in defined cell types.

In summary, we could demonstrate that TGF-β elicits potent growth inhibitory effects in primary mouse VSMCs, in the absence of any pro-apoptotic effects. Multiple kinase systems and transcription factors are activated by TGF-β in VSMCs, including Smad2/3, p38, p42/44, and JNK, but p38 plays an essential and nonredundant role in TGF-β-dependent growth inhibition. Modulation of the p38 pathway may therefore serve as an attractive target to modify phenotypic switching of VSMCs in angiogenesis, atherosclerosis, or restenosis, conditions characterized by VSMC plasticity and proliferation.

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References


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