Smad6s Regulates Plasminogen Activator Inhibitor-1 through a Protein Kinase C-β-dependent Up-regulation of Transforming Growth Factor-β*

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David T. Berg‡, Laura J. Myers‡, Mark A. Richardson, George Sandusky, and Brian W. Grinnell§
From the Division of Biotechnology Discovery Research, Lilly Research Laboratories, Indianapolis, Indiana 46285

Plasminogen activator inhibitor-1 (PAI-1) is a serpin class protease inhibitor that plays a central role in the regulation of vascular function and tissue remodeling by modulating thrombosis, inflammation, and the extracellular matrix. A central mediator controlling PAI-1 is transforming growth factor-β (TGF-β), which induces its expression and promotes fibrosis. We have found that a unique member of the Smad family of signal transduction molecules, Smad6s, modulates the expression of PAI-1. Overexpression of Smad6s in endothelial cells increases promoter activity and PAI-1 secretion, and an antisense to Smad6s suppresses the induction of PAI-1 by TGF-β. The effect of Smad6s on the PAI-1 promoter appeared to be the result of increased binding of the forkhead winged helix factor FoxD1 to a TGF-β-responsive element. Furthermore, the effect of Smad6s on PAI-1 up-regulation and on FoxD1 binding was found to result from up-regulation of TGF-β and could be inhibited by the blocking TGF-β signaling with Smad7. The ability of Smad6s to regulate the TGF-β promoter and subsequent PAI-1 induction was suppressed by a selective protein kinase C-β (PKC-β) inhibitor. Consistent with the in vitro data, we found that increased Smad6s in diseased vessels correlated with increased TGF-β and PAI-1 levels. Overall, our results demonstrate that the level of Smad6s can alter the level of TGF-β and the subsequent induction of PAI-1 via a FoxD1 transcription site. Furthermore, our data suggest that this process, which is up-regulated in diseased vessels, can be modulated by the inhibition of PKC-β.

Vascular injury plays a major role in the pathogenesis of multiple acute and chronic disorders. The response to initial injury triggers activation of coagulation, inflammatory and repair processes aimed at maintaining vascular integrity; however, an imbalance in this process can ultimately lead to aberrant fibrosis, structural dysfunction, and ultimately chronic disease. While many factors play a role in the continuum from acute injury to chronic dysfunctional vasculature, the serpin plasminogen activator inhibitor-1 (PAI-1),† has been shown to play a key role in the pathogenesis of both acute and chronic disorders, including cardiovascular, renal, hepatic, and pulmonary (reviewed in Refs. 1–4).

As the physiologic inhibitor of tissue-type plasminogen activator, PAI-1 regulates the conversion of plasminogen to plasmin and thus plays a critical role in regulating the balance between coagulation and fibrinolysis. In general, the plasminogen activator/plasmin pathway has been implicated in a wide variety of physiologic and pathologic processes that require tissue remodeling and cell motility (5). As such, PAI-1 influences a number of cellular processes (including wound repair, cell migration, angiogenesis, neointima formation, and matrix-dependent cell attachment) largely via interactions with urokinase plasminogen activator receptor, vitronectin, and indirectly through plasmin activation of extracellular matrix proteases and latent growth factors (reviewed in Ref. 6). While an important component in response to injury, chronically elevated plasma PAI-1 has been shown in increase risk for thrombosis, cancer metastasis, vascular complications of diabetes, and the development of septic shock (7–9).

Although a number of cytokines and hormones are known to regulate PAI-1 (reviewed in Ref. 10), a major factor controlling PAI-1 synthesis is transforming growth factor-β (TGF-β). Both TGF-β1 and TGF-β3 have been shown to up-regulate PAI-1 in cultured human endothelial cells at the promoter level (11–14), and although the endothelial regulation in vivo has not been widely studied, Dong et al. (15) have shown that endothelial PAI-1 up-regulation in human aortic allografts is mediated mainly by the TGF-β pathway. TGF-β family signaling is positively modulated by various members of the Smad family of signal transduction proteins (reviewed in Refs. 16 and 17), however, two Smads (Smad6 and 7) inhibit TGF-β signal transduction. The Smad7 protein has been extensively studied and shown to prevent phosphorylation of receptor-activated Smads, thereby inhibiting TGF-β-induced signaling responses (18, 19). Smad6 has been shown to inhibit signaling by the TGF-β superfamily (20, 21), and to play a role in the development of the cardiovascular system (22). However, an endothelial splice variant of Smad6, designated Smad6s, recently has been shown to have differential activity relative to the inhibitory Smad6 and Smad7 in a Xenopus model, being an antagonist of the BMP pathway but an agonist of the activin pathways (23), and showing enhanced modulation of TGF-β signaling in human endothelial cells (24).

In this study, we have explored the role of TGF-β signaling in controlling the expression of PAI-1 in human endothelial cells, focusing on the role of the endothelial Smad6s. Using overexpression and antisense modulation, we show that Smad6s regulates the expression and secretion of PAI-1 by enhancing the

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‡ Both authors contributed equally to this work.
§ To whom correspondence should be addressed: Biotechnology Discovery Research, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285-0444. Tel.: 317-376-2293; Fax: 317-277-2954; E-mail: bgrinnell@lilly.com.
† The abbreviations used are: PAI-1, plasminogen activator inhibitor-1; BMP, bone morphogenetic protein; CAT, chloramphenicol acetyltransferase; TGF-β, transforming growth factor-β; ELISA, enzyme-linked immunosorbent assay; PKC, protein kinase C.
binding of nuclear factor FoxD1 to a previously identified TGF-β-responsive element in the promoter. Moreover, we demonstrate the up-regulation of PAI-1 synthesis by Smad6s depends on an induction of TGF-β itself and that this process can be effectively modulated by a selective PKC-β inhibitor. Furthermore, we show that in human diseased vessels overexpressing Smad6s, both TGF-β1 and PAI-1 are overexpressed, consistent with the up-regulation observed in vitro.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cell Culture—TGF-β1 and TGF-β3 were purchased from R&D Systems. SVHA-1 cells, an SV40-transformed human aortic endothelial cell line, were described previously (24). Human umbilical vein endothelial cells were obtained from Clonetics (San Diego, CA). The ECV304 cell line was obtained from the ATCC (CRL-1998) and grown as described previously (25). Cells were maintained in Dulbecco’s modified Eagle’s medium/F-12 (3:1), a medium composed of a 3:1 v/v mixture of Dulbecco’s modified Eagle’s medium and Ham’s nutrient mixture F-12. Dulbecco’s modified Eagle’s medium/F-12 (3:1) and fetal bovine serum were purchased from Invitrogen. The basal medium was supplemented with 10 mM L-glutamine, 50 μM 2-aminomethanol, 20 mM HEPES, 50 μg/ml gentamycin, and 5% fetal bovine serum. The PKC inhibitor LY379196 was from Eli Lily and Co. All other reagents were of the highest quality available.

**DNA Transfection, Chloramphenicol Acetyltransferase (CAT) ELISA, Luciferase, and PAI-1 Assays—**The vector pOCAT2336 containing the PAI-1 promoter and the Smad6s and Smad7 expression vectors were described previously (23). The TGF-β-responsive artificial PAI-1 reporter plasmid p3TP-lux was originally cloned by Dr. J. Massague’s laboratory (26). Endothelial cells were seeded in 6-well plates to 80% confluence. DNA was transfected at a concentration of 1 μg for pOCAT2336 and p3TP-lux and 5 μg for the Smad vectors with Invitrogen’s Lipofectin reagent used according to manufacturer’s instructions. Expressed CAT protein was measured using a CAT ELISA kit from Oncogene Science and performed according to manufacturer’s protocol. The plates were read kinetically and data expressed in milliunit optical density units/min. The activity of firefly luciferase was measured by a Dynax microtiter plate luminometer. PAI-1 antigen concentration in the conditioned medium was measured by a TintElis kit (Biopool International) according to the manufacturer’s instructions.

**Antisense Oligodeoxynucleotides—**The oligonucleotides used in antisense experiments were synthesized with phosphorothioates and C-5 labelled oligonucleotides following standard protocols (27). Antisense oligodeoxynucleotides (oligos) were designed to hybridize to the region of the Smad6s mRNA encompassing the initial ATG. The sequence of the antisense oligodeoxynucleotide for Smad6s was 5’-GGTTGCGCCAT-TCTGGAACAT-3’, and the sense strand control was 5’-ATGTCGCAAGAT- GGCGAACACC-3’. Oligos were introduced to cells following the protocol of Sandusky et al. (28). After 4 days each experimental condition was treated with or without TGF-β at a concentration of 1 ng/ml in the maintenance medium. The antisense was shown to inhibit the Smad protein levels by Western blot analysis.

**TGF-β Promoter Activation and Secretion—**TGF-β1 (28) and -β2 promoter (29) constructs driving CAT expression (1 μg) were co-transfected with Smad6s vector (5 μg), Smad7 (5 μg), or with a control pCMV neo vector (Promega, Madison, WI). Transfection with Lipofectin and assay for TGF-β1 and TGF-β3 by ELISA were as described previously (24). CAT activity expression was measured kinetically. Lysates were normalized using a BCA assay measuring total protein concentration.

**Immunohistochemistry—**Tissue specimens were retrieved from the tissue bank of Lilly Research Laboratories. These tissues were obtained from the Cooperative Human Tissue Network using an institutional review board-approved protocol. All human samples were derived from surgical specimens obtained during the period extending from 1996 to 1999. Tissues were fixed overnight in zinc-buffered formalin and then transferred to 70% ethanol prior to processing through paraffin. The sectioning and staining with antibodies against Smad6, TGF-β, and PAI-1 were as described previously (24). A biotinylated secondary antibody plus streptavidin-horseradish peroxidase kit (Dako LSAB2) was then utilized along with a DAB chromagen and peroxide substrate to detect the bound antibody complexes. The slides were briefly counterstained with hematoxylin and reviewed by two investigators (G. E. Sandusky and M. Donovan) using light microscopy to evaluate the intensity and localization of the staining.

**RESULTS**

**TGF-β Enhances PAI-1 Expression from Human Endothelial Cells—**As shown in Fig. 1A, the level of PAI-1 secreted from human SVHA-1 endothelial cell line was increased following treatments with TGF-β1 (and TGF-β3; data not shown). In both endothelial SVHA-1 cells and in the ECV304 line, the effect was dose-dependent with a half-maximal response of ~25 pg. Moreover, using both the basal PAI-1 promoter- and TGF-β-sensitive 3TP-lux plasmid (Fig. 1B), we observed significant up-regulation of the promoter level by both TGF-β1 and TGF-β3 (data not shown) in these cell lines. As indicated above, endothelial Smad6s has been shown to differentially regulate TGF-β family signaling, and we sought to determine the role of...
this regulator on PAI expression. As shown in Fig. 1C, an antisense that blocked Smad6s, but not a sense control, significantly reduced the secretion of PAI-1 by TGF-β.

Smad6s Enhances TGF-β Activation of PAI-1 and Nuclear Factor FoxD1—The antisense data suggested that PAI-1 upregulation was influenced by the presence of endogenous Smad6s. We confirmed this by overexpressing Smad6s and examining its affect on PAI-1 secretion and promoter activity. As shown in Fig. 2A, the overexpression of Smad6s resulted in an increase in both endogenous PAI-1 mRNA and in the secretion of PAI-1. Using both the basal promoter- and TGF-β-sensitive 3TP-lux indicator plasmid in co-transfection experiments, overexpression of Smad6s resulted in an increase in PAI-1 promoter activity (Fig. 2B), suggesting that the effect on endogenous secretion and mRNA was the result of promoter activation.

The p3TP-lux promoter contains nucleotides −740 to −636 of the PAI-1 promoter region, and previous studies have suggested several regions important for TGF-β regulation, including the region from −726 to −704 (32). Using an electrophoretic mobility shift assay, we have identified nuclear binding to a site in this region for the forkhead winged helix factor FoxD1 (Fig. 3A). The binding was specific and could be competed with cold binding site, and a mutation in the core FoxD1 sequence resulted in poor binding. Moreover, the overexpression of Smad6s was able to enhance the binding of FoxD1 to this region of the PAI-1 promoter, which corresponded with an increase in PAI-1 secretion from the treated cells (Fig. 3B). In addition, the binding of FoxD1 to the promoter region of PAI-1 could be increased by TGF-β alone (Fig. 3C).

Smad6s Induction of TGF-β and Suppression by PCK-β Inhibition—As shown above, the overexpression of Smad6s induces PAI-1 and the binding of FoxD1 to the previously identified TGF-responsive site in the PAI-1 promoter, i.e. Smad6s appears to mimic the TGF-β response at the PAI-1 promoter. If the effect of Smad6s on increasing FoxD1 binding were dependent on TGF-β signaling, then we would expect inhibition of TGF-β receptor signaling by Smad7 to inhibit binding. As shown in Fig. 4A, both the increase in FoxD1 binding and the subsequent increase in PAI-1 secretion were inhibited by the overexpression of inhibitory Smad7. In addition, we examined the effect of overexpressing Smad6s on TGF-β levels secreted into the culture medium. As shown, Smad6s overexpression was capable of increasing the amount of TGF-β1 secreted from the cell (Fig. 4B). Moreover, the effect on TGF-β appeared to be the result of increased expression as indicated by the ability of Smad6s to induce both the TGF-β1 and TGF-β3 promoters.

Previous studies have suggested that TGF-β mRNA induction can be sensitive to PKC inhibition (33, 34). To determine...
whether the Smad6s regulation was kinase-dependent, we treated cells with staurosporin at concentrations known to inhibit PKC and found complete inhibition of the Smad6s regulation of TGF-β (Fig. 4B). Moreover, using the PKCβ-selective inhibitor LY379196 the effect of Smad6s also could be completely eliminated.

**Differential Regulation of Smad6s, PAI-1, and TGF-β in Vascular Disease**—The data above suggested that the level of Smad6s in endothelial cells could alter the level of TGF-β and the subsequent induction of PAI-1. To determine whether this relationship was observed in endothelial cells in vivo, we examined normal and atherosclerotic human cardiovascular tissues with Smad6s-, TGF-β1-, and PAI-1-specific antibodies, as studies have previously linked high PAI-1 levels to fibrotic vascular disease and also have demonstrated TGF-β overexpression in fibroproliferative vascular lesions (35) and lipoid-rich aortic intimal lesions (36). In an analysis of 35 CD34-positive vascular samples, Smad6s levels were very low to undetectable in normal vessels but overexpressed in the endothelium over the plaque, as well as in the plaque vasculature (Fig. 5). Whereas little if any staining could be observed in non-diseased vessels with the TGF-β1- and PAI-1-specific antibodies, high levels of expression were observed in the Smad6s-positive vessels. These data support the in vitro observations in cultured cells and are consistent with Smad6s playing a role in regulating TGF-β and PAI-1 in vivo.

**DISCUSSION**

Under normal circumstances, the vascular endothelium displays a number of regulatory mechanisms that modulate coagulation, inflammation, and vascular function to maintain a hemostatic balance in the local environment (37, 38). As this balance is disturbed by either acute or chronic insult, pathological processes ensue that alter vascular function and ultimately organ function. Both PAI-1 and TGF-β play important roles in modulating the response to vascular and tissue injury, but factors that enhance or chronically elevate levels of TGF-β and consequently PAI-1 compromise vascular function and promote pathologic processes. As such, understanding the coordinate regulation of TGF-β and PAI-1 may provide the basis for improved targets for developing therapeutic intervention. In this paper we provide new mechanistic understanding for the control of vascular PAI-1 and TGF-β through the regulatory factor Smad6s and further demonstrate that the coordinate regulation of PAI-1 by Smad6s induction of TGF-β can be modulated by isozyme-specific inhibition of PKCβ.

As reviewed above, the endothelial splice variant Smad6s has been shown to differentially regulate TGF-β family members in *Xenopus* (23) and to positively modulate TGF-β suppression of endothelial thrombomodulin (24). As shown in Fig. 4, Smad6s functions to increase the expression and subsequent secretion of TGF-β. Interestingly, Smad6 mRNA itself has been reported to be induced by TGF-β1 and BMP-7 (39, 40), and BMP-7 has been shown to regulate the expression of PAI-1. While further studies will be required to define these relationships, these observations along with the data presented here suggest that the control of TGF-β/Smad6-related functions form an intricate feedback loop regulating TGF-β levels and the induction of PAI-1.

Several studies have identified the regions in the 5′-flanking region of human PAI-1 gene from −799 and −82 as mediating up-regulation of reporter gene expression by TGF-β1 in endothelial cells, both in vitro and in vivo (41). More specifically the region −791 to −546 upstream of the PAI-1 gene cap site (42) and from −740 to −636 (43) have been shown to confer response to TGF-β. Most notable, a major TGF-β-responsive element was identified by DNA footprinting to bind a factor in the region −726 to −703 (32), although at this time this region did not contain consensus sequences for any known transcription factors. Studies by Kutsa et al. (44) recently identified this same general region, from nucleotides −740 and −703, as required for serum response. Our data showed that Smad6s overexpression increased nuclear factor binding to a FoxD1 site centered at −720. The forkhead transcription factor FoxD1, also called FREAC-4 (45) or murine BF-2, appears to play a role in developmental patterning and in kidney morphogenesis/nephrogenesis (46–48). While this study is the first to define a role for FoxD1 in TGF-β response, the xenopus homolog XBF2 has been shown to modulate signaling of another TGF-β family member BMP-4 (49). Of interest, the FoxD1 site is adjacent to a Smad3/Smad4 CAGA box/AGAC site (50, 51), a site shown to confer TGF-β stimulation when multimerized in a heterologous reporter construct; future studies will be needed to determine whether Smad3/4 interacts with FoxD1 following its induction and binding to this region. Hou et al. (52) recently showed that the upstream regulatory region of PAI-1 is extended up to 15 kb, suggesting that future analysis of PAI-1 regulation will need to extend beyond the proximal promoter.

Several studies have shown that both PAI-1 and TGF-β can be regulated by modulating PKC. Using cells deficient in PKCβ, previous studies have shown that PAI synthesis is dependent on PKC-β (53) and that the activation of PKC-β may mediate the production of PAI-1 in endothelial cells (54). In addition, the PKCβ-specific inhibitor LY379196 effectively prevented low density lipoprotein-induced PAI-1 production (54) as well as PAI-1 mRNA induction via vβ3 (55). In models of diabetes, studies have shown that TGF-β mRNA expression can be modulated by a PKCβ-specific inhibitor (33, 34), and the induction of TGF-β by cigarette smoke could be significantly suppressed by LY379196, suggesting PKCβ involvement (56). Our data now supply a possible link to these observations of PKCβ dependence via modulation of Smad6s.

Overall, our data provide new mechanistic understanding for the control of PAI-1 by TGF-β. The results presented here suggest that Smad6s plays a prominent role in controlling the TGF-β-dependent expression of PAI-1 and that targeting this Smad signal may provide new opportunities for therapeutic intervention in treating and preventing vascular dysfunction. Specifically, our data give a direct link to previous observations
on PKC-β modulation and provide support for this kinase as a target for modulating vascular function where dysregulation of both TGF-β and PAI-1 is important to the pathophysiology of the disorder. While our data give new mechanistic understanding for TGF-β regulation, further studies will be needed to determine the relationship of these findings to other important mediators (57, 58) of PAI-1 regulation.

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