Muscle Cell Activation Is Mediated via Smad3*

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Activating vascular smooth muscle cells (VSMCs) by proinflammatory cytokines is a key feature of atherosclerotic lesion formation. Transforming growth factor (TGF)-β1 is a pleiotropic growth factor that can modulate the inflammatory response in diverse cell types including VSMCs. However, the mechanisms by which TGF-β1 is able to mediate these effects remains incompletely understood. We demonstrate here that the ability of TGF-β1 to inhibit markers of VSMC activation, inducible nitric-oxide synthase (iNOS) and interleukin (IL)-6, is mediated through its downstream effector Smad3. In reporter gene transfection studies, we found that among a panel of Smads, Smad3 could inhibit iNOS induction in an analogous manner as exogenous TGF-β1. Adenoviral overexpression of Smad3 potently repressed inducible expression of endogenous iNOS and IL-6. Conversely, TGF-β1 inhibition of cytokine-mediated induction of iNOS and IL-6 expression was completely blocked in Smad3-deficient VSMCs. Previous studies demonstrate that CCAAT/enhancer-binding protein (C/EBP) and NF-κB sites are critical for cytokine induction of both the iNOS and IL-6 promoters. We demonstrate that the inhibitory effect of Smad3 occurs via a novel antagonistic effect of Smad3 on C/EBP DNA-protein binding and activity. Smad3 mediates this effect in part by inhibiting C/EBP-β and C/EBP-δ through distinct mechanisms. Furthermore, we find that Smad3 prevents the cooperative induction of the iNOS promoter by C/EBP and NF-κB. These data demonstrate that Smad3 plays an essential role in mediating TGF-β1 anti-inflammatory response in VSMCs.

Accumulating evidence suggests that atherosclerosis is a chronic inflammatory disease state. The lesions of atherosclerosis represent a series of complex interactions between immune (macrophage/T lymphocyte) and non-immune (endothelial cells/VSMCs) cells (1). Although the initial phase of atherogenesis involve endothelial activation and immune cell recruitment, the evolution of this lesion is critically modulated by VSMCs. Through effects on VSMC migration, proliferation, and elaboration of cytokines and extracellular matrix proteins, proinflammatory stimuli can alter atherosclerotic lesion characteristics and attendant complications (1, 2). Identification of mechanisms that limit the VSMC response to proinflammatory stimuli is, thus, of considerable interest.

CCAAT/enhancer-binding proteins (C/EBPs) constitute a family of transcription factors that can promote the inflammatory response in VSMCs. C/EBPs contain an activation domain, a DNA binding basic region, and dimerization domain and may bind to DNA as either homodimers or heterodimers (3, 4). Among the C/EBPs expressed in VSMCs, only C/EBP-β and C/EBP-δ are induced in response to inflammatory stimuli such as IL-1β (5). In addition, C/EBP-β and C/EBP-δ can directly regulate pro-inflammatory gene expression at the level of transcription alone or through the cooperativity with NF-κB in several cell types including VSMCs (3, 6–8). For example, both NF-κB and C/EBP sites are required for IL-1β-mediated induction of the iNOS and IL-6 promoters in rat aortic smooth muscle cells (RASMCs) (7, 8). C/EBPs can also contribute to the inflammatory response under different pathophysiological conditions. For instance, C/EBP-β can mediate hypoxia-induced iNOS expression in rat pulmonary smooth muscle cells (8). Furthermore, inhibition of C/EBP DNA binding through the use of decoy oligonucleotides decreases vascular lesion formation in a rabbit model of restenosis (9). Thus, identification of mechanisms or signaling pathways to inhibit C/EBP-β or C/EBP expression may offer novel strategies to diminish the inflammatory response in VSMCs for diverse vascular occlusive disease states.

Transforming growth factor (TGF)-β1 is a pleiotropic growth factor involved in cell growth, differentiation, and immune modulation (10, 11). The composite anti-proliferative and anti-inflammatory effects of TGF-β1 on both the immune and non-immune cellular constituents of the atherosclerotic lesion suggest an inhibitory role in atherogenesis. Indeed, blockade of TGF-β1 ligand or the TGF-β type II receptor accelerates the development of atherosclerotic lesion formation in apoE−/−/atherosclerotic-prone mice (12, 13). In patients, the serum concentration of active TGF-β1 is inversely correlated with the severity of atherosclerotic disease (14). However, the mecha-
nism by which TGF-β1 is able to mediate these effects on VSMCs remains poorly understood.

Recently, a class of proteins termed Smads have been identified as the downstream effectors of TGF-β1 signaling (10, 11, 15). We hypothesized that members of the Smad family may be involved in mediating TGF-β1 inhibition of VSMC activation in response to proinflammatory stimuli. In this report we demonstrate both gain- and loss-of-function experiments that Smad3 is essential in inhibiting markers of VSMC activation, iNOS and IL-6, after stimulation with IL-1β. Furthermore, our studies suggest that the Smad3 inhibition occurs through distinct effects on C/EBP-β and C/EBP-α.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—RASMCs were harvested from the thoracic aortas of adult male Sprague-Dawley rats (200–250 g; Zivic-Miller Co., Zelienople, Pa) by enzymatic digestion according to the method of Gunther et al. (16). 293T cells were obtained from Dr. Hamid Band (New England Medical Center, Boston, MA) and maintained as described (17). Mouse aortic smooth muscle cells (MASMCs) were isolated from the aortas of Smad3 wild-type (wt) or Smad3-knockout mice (age 6–8 weeks old) essentially as described previously (18) and characterized by smooth muscle α-actin and calponin immunostaining (data not shown). Smad3−/− mice were provided by X-F Wang (Duke University) (19). Animal care and procedures were approved by the Harvard Medical School Standing Committee on Animals and were performed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care (NIH). We obtained IL-1β and active recombinant TGF-β1 from R&D Systems.

RNA Extraction and RNA Blot Analysis—RNA was isolated from cultured cells by guanidine isothiocyanate extraction and centrifugation through cesium chloride. RNA was fractionated on a 1.3% agarose gel and transferred to nitrocellulose filters. The filters were hybridized with 32P-labeled, random-primed cDNA probes, washed, and exposed as described previously (20). A cDNA probe for IL-6 was generated from RAW264.7 DNA using standard polymerase chain reaction methods with the following primers: upper, 5′-GTTGCGCCTTCTTGGGACTGT-3′, and lower, 5′-ATGAGTTGGATGTCCTTGGTT-3′. cDNA probes for C/EBP-β and C/EBP-α were obtained as gifts from S. McKnight (Dallas, TX).

Transient Transfections and Adenoviral Infection—RASMCs were transfected with PaGENE 6 transfection reagent (Roche Applied Science) as described (20). The total amount of plasmid DNA was kept constant within each experiment using pcDNA3. Luciferase activity was normalized to β-galactosidase activity by co-transfecting the pCMV-β-gal plasmid in all experiments. All transfections were performed in triplicate from at least three independent experiments. The expression plasmids for Smad1, -2, -3, -4, and -7 have been described previously (20) and were expressed at comparable levels as verified by immunoblotting (data not shown). The mouse −1.5-kb iNOS promoter construct was obtained from C. Glass (San Diego, CA) (21), and the mouse −250-bp IL-6 promoter construct was obtained from R. Schwartz (Ann Arbor, MI). For adenoviral infection of RASMCs, replication-deficient human adenovirus vectors expressing mouse Smad3 or LacZ (encoding for β-galactosidase) under the control of the cytomegalovirus promoter (Ad-Smad3 or Ad-β-gal) were used (a gift from K. Miyazono, Tokyo). Cells seeded at 2 × 10⁵/10-cm² dish were infected with the adenoviral vectors at 100 multiplicity of infection and incubated for 16 h before treatment with IL-1β or TGF-β1 as indicated.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays—Nuclear extracts from RASMCs were prepared, and mobility shift analyses were performed as previously described (20). DNA probes were generated to the C/EBP element at position −927 to −906 bp of the human iNOS promoter as double-stranded oligonucleotides corresponding to the wild-type sequence, 5′-CAATTATCTGCTACGTCTCAGTCATTCTTT-3′, or mutated sequence, 5′-CAATTATTCTGCTACGTCTCATTCTTT-3′. Supershift antibodies for C/EBP-β (sc-150X), C/EBP-α (sc-151X) (Santa Cruz Biotechnology), or IgG1 control antibody (Sigma) were incubated with nuclear extracts for 2 h at 4 °C before adding the radiolabeled oligonucleotide.

Western Blot Analyses and Immunoprecipitation Studies—Western blot analyses were performed as described (20). Immunoblots were incubated with the appropriate primary antibodies for Smad3 (Zymed Laboratories Inc., South San Francisco, CA), C/EBP-β (sc-150) or C/EBP-δ (sc-151) (Santa Cruz Biotechnology, Santa Cruz, CA), or the FLAG epitope tag (Sigma) and were detected using horseradish peroxidase secondary antibodies and chemiluminescence. For immunoprecipitation assays, 293T cells were transfected with the indicated expression plasmids and harvested with radioimmune precipitation assay buffer 48 h later. Lysates were subjected to immunoprecipitation with either 4 μg of α-FLAG M2 monoclonal antibody (Sigma) or 4 μg of IgG1 control antibody (Sigma) at 4 °C for 2 h followed by incubation with protein A/G-Sepharose beads overnight at 4 °C. The beads were washed, and proteins were separated by SDS-PAGE as previously described (22).

RESULTS

TGF-β1 Can Inhibit Cytokine-induced Expression of iNOS and IL-6 in VSMCs—Stimuli such as IL-1β can activate iNOS and IL-6 expression by stimulating members of the C/EBP and NF-κB families (8, 23–25). To examine whether TGF-β1 can inhibit inducible expression of iNOS and IL-6 mRNA in VSMCs, RASMCs were stimulated with IL-1β (10 ng/ml) in the presence or absence of pretreatment with active TGF-β1 (10 ng/ml) for 30 min. As demonstrated in Fig. 1A, iNOS and IL-6 mRNA are expressed at low levels in unstimulated cells and induced markedly by IL-1β. Pretreatment with TGF-β1 potently attenuated this induction (Fig. 1A). Previous work has demonstrated that IL-1β induction of both iNOS and IL-6 occurs primarily at the level of transcription (8, 23–25). Consistent with these results, transient transfection assays using iNOS and IL-6 promoter constructs showed an increase in transcriptional activity induced by IL-1β; however, pretreatment with TGF-β1 for 30 min markedly attenuated this response in RASMCs (Fig. 1B).

Cellular signaling from the TGF-β superfamily occurs through the intracellular signaling mediators, termed Smads,
which translocate to the nucleus, where they direct transcriptional responses. Three classes of Smads, pathway-restricted, common, and inhibitory, are responsible for coordinating the downstream signaling effects (26) (11, 27). TGF-β/activin receptors phosphorylate the pathway-restricted Smads, Smad2 and Smad3, whereas bone morphogenic protein receptors activate Smads 1, 5, and 8. Upon phosphorylation, these pathway-restricted Smads may hetero-oligomerize with Smad4, the only common Smad, and translocate to the nucleus where they may participate in regulating transcriptional events. Smad6 and Smad7, known as inhibitory Smads, are structurally divergent from other Smads and function to block TGF-β signaling by preventing activation of pathway-restricted Smads (26). To assess whether TGF-β1 inhibition was Smad-dependent in VSMCs, we first performed cotransfection assays on the iNOS promoter construct with several Smad expression constructs followed by stimulation with IL-1β. As shown in Fig. 1C, of the pathway restricted Smads, only Smad3 was able to repress the iNOS promoter in a manner similar to TGF-β1, an effect enhanced by treatment with exogenous TGF-β1. In contrast, the addition of TGF-β1 to Smad2 had no further inhibitory effect in comparison to TGF-β1 alone. The common Smad, Smad4, could also inhibit the induction of the iNOS promoter, albeit much weaker than Smad3 alone. Taken together, these data indicate that in response to TGF-β, Smad3 may mediate the inhibitory effect of TGF-β1 in VSMCs.

**Smad3 Is Requisite for Mediating TGF-β1 Inhibition on IL-1β-induced Expression of iNOS and IL-6 in VSMCs**—To assess whether constitutive overexpression of Smad3 can inhibit inducible expression of endogenous iNOS and IL-6 in VSMCs, we adenovirally overexpressed Ad-Smad3 or Ad-cytomegalo virus-β-galactosidase and treated the cells in the presence of Ctrl (water) or IL-1β (10 ng/ml). As shown in Fig. 2A, IL-1β-induced iNOS and IL-6 mRNA; however, this induction is markedly attenuated upon adenoviral overexpression of Smad3. To assess whether Smad3 is requisite in mediating the TGF-β1 inhibitory effect on iNOS and IL-6 in VSMCs, we isolated MASMCs from the aortas of Smad3-wt or Smad3-deficient (ko) mice. By Western analysis, we verified the absence of Smad3 protein in Smad3-ko MASMCs (Fig. 2B). Total RNA was harvested from Smad3-wt or Smad3-ko MASMCs 24 h after stimulation with Ctrl (water), IL-1β, IL-1β plus TGF-β1, or TGF-β1 alone. As shown in Fig. 2C, TGF-β1 inhibited the IL-1β-mediated induction of iNOS and IL-6 in Smad3-wt MASMCs; however, TGF-β1 inhibition is dramatically blocked in Smad3-ko MASMCs. These data suggest a critical role for Smad3 in mediating TGF-β1 inhibition of these pro-inflammatory markers in VSMCs.

**Smad3 Inhibits Cytokine-induced C/EBP DNA-Protein Binding and Prevents C/EBP and NF-κB Cooperativity in VSMCs**—As a first step to investigate the mechanism by which Smad3 mediates the inhibitory effect on iNOS expression we examined the iNOS promoter. Previous studies demonstrate that in VSMCs induction of iNOS is dependent upon both the proximal NF-κB and C/EBP sites (8, 28). To assess whether TGF-β1 or Smad3 can affect either NF-κB or C/EBP DNA-protein binding we performed gel shift studies using nuclear extracts from RASMCs. Consistent with previous reports, treatment with TGF-β1 (29) or adenoviral overexpression of Smad3 (Adv-Smad3) had no effect on IL-1β-induced NF-κB DNA binding (data not shown). In contrast, in response to TGF-β1, Adv-Smad3 markedly reduced C/EBP DNA-protein binding (Fig. 3A). Specificity of the induced C/EBP complex was verified by competition studies with unlabeled wild-type or mutant oligonucleotides (Fig. 3B). Furthermore, supershift studies verified the presence of C/EBP-β and C/EBP-δ within this induced complex (Fig. 3C).

The NF-κB transcription factor complex is composed of variable subunits p50, p52, p65(ReLa), c-Rel, and RelB (30). Previous studies support a direct cooperation between NF-κB and C/EBP on the iNOS promoter. To assess whether TGF-β1 alters C/EBP DNA binding or the cooperative activation of iNOS by C/EBP and NF-κB transcription factors, we performed co-transfection studies in the presence of C/EBP-β, C/EBP-δ, or p50. In response to a constitutively active TGF-β type I receptor, Smad3 cannot inhibit the induction of iNOS by C/EBP-β and C/EBP-δ alone or in combination with NF-κB p50 (Fig. 3D). Consistent with the modest inhibitory effect by the common Smad, Smad4 (Fig. 1C), the addition of Smad4 to Smad3 enhanced the inhibition of C/EBP-β and C/EBP-δ or their cooperativity with NF-κB p50 on the iNOS promoter (Fig. 3D). Taken together, these data indicate that Smad3 alone or in combination with Smad4 can prevent the induction of C/EBP DNA binding or the cooperative activation of iNOS by C/EBP-β and NF-κB.

**TGF-β1/Smad3 Inhibits C/EBP-β and C/EBP-δ through Distinct Mechanisms**—In theory, inhibition of C/EBP DNA binding could be the result of a decrease in the levels of C/EBP expression, a direct interaction of Smad3 with C/EBP-β or C/EBP-δ or both. To assess whether TGF-β1 alters C/EBP-β or C/EBP-δ expression, we treated MASMCs with either vehicle (control), IL-1β, IL-1β plus TGF-β1, or TGF-β1 alone. As shown in Fig. 4A, TGF-β1 had minimal effect on IL-1β-induced C/EBP-β expression, whereas induction of C/EBP-δ was markedly inhibited. Although IL-1β induction of C/EBP-β expression is minimally affected by TGF-β1, C/EBP DNA binding is
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Smith3 interacts with C/EBP and inhibits VSMC activation.

**Discussion**

Inflammation is a critical hallmark in the development of a variety of vascular occlusive disease states. VSMCs are an important source of inflammatory mediators that may participate directly in atherogenesis or prognosticate future cardiovascular events. Thus, identification of mechanisms to limit the inflammatory response may offer novel therapeutic targets. One of the established functions of TGF-β1 is its ability to inhibit activation in both immune and non-immune cells. Studies in our laboratory and others demonstrate that TGF-β1 can have cell type-specific effects, we sought to examine the mechanism by which TGF-β1 mediated its inhibitory effect in VSMCs.

In this report, we provide evidence that Smad3 is a critical effector in TGF-β1-mediated inhibition of VSMC activation. In support, we found that Smad3 could inhibit the expression of markers of VSMC activation (Fig. 1). The addition of exogenous TGF-β1 enhanced the Smad3-mediated inhibition. Furthermore, although constitutive overexpression of Smad3 inhibited the inducible expression of endogenous iNOS and IL-6 in VSMCs, Smad3-deficient VSMCs completely abolished the TGF-β1-mediated inhibition (Fig. 2). The more potent inhibitory effect of TGF-β1 achieved on cytokine-induced iNOS and
with Smad4. We similarly observed enhanced inhibition with Smad3 and Smad4 on the iNOS promoter in RASMCs (Fig. 3D). Although our observations in Smad3-deficient MASMCs indicate that Smad4 is dispensable for mediating the TGF-β1 inhibition (Fig. 2), Smad4 can hetero-oligomerize with Smad3 to facilitate its translocation into the nucleus. Thus, in response to TGF-β, Smad4 may be able to potentiate Smad3 inhibitory effect. A recent study that examined the role of Smad3 in adipocyte differentiation, a C/EBP-β- and C/EBP-δ-regulated process, also demonstrated an inhibitory effect on these C/EBPs during 3T3-L1 differentiation (34). Interestingly, Smad3 had no effect on C/EBP DNA binding, but it did affect the transcriptional activity of C/EBP-β and C/EBP-δ (34). The discrepancy between the effects of Smad3 on C/EBP DNA binding observed in this study and ours is unclear but may be secondary to cell type-specific effects mediated by TGF-β1 in 3T3-L1 cells versus RASMCs or the absence of cytokines for C/EBP induction in the 3T3-L1 system.

Taken together, our observations using both gain- and loss-of-function strategies indicate that Smad3 plays a critical role in modulating VSMC activation by TGF-β1. Furthermore, we provide evidence that the Smad3 inhibition occurs in part through disrupting C/EBP DNA binding by distinct inhibitory effects on C/EBP-β and C/EBP-δ. In addition, Smad3 prevents the cooperative induction by C/EBP and NF-κB on markers of VSMC activation. Because C/EBPs are essential for the inducibility and transcriptional activation of several other pro-inflammatory and acute-phase proteins, these findings may have broad implications on the understanding of the mechanism by which TGF-β1 limits the inflammatory response in VSMCs.

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