Changes in Smad expression and subcellular localization in bleomycin-induced pulmonary fibrosis

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Venkatesan, N., L. Pini, and M. S. Ludwig. Changes in Smad expression and subcellular localization in bleomycin-induced pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 287: L1342–L1347, 2004.—Administration of bleomycin (BM) produces inflammation and fibrosis of the lung in humans and experimental animals. The molecular defects by which BM induces these pathological effects have not been studied in detail. We studied the expression of Smad family proteins, key molecules involved in mediating transforming growth factor-β (TGF-β) signaling from the cell membrane to the nucleus, during the early and late phases of BM-induced fibrogenesis. Pulmonary fibrosis was induced in male Sprague-Dawley rats by a single intratracheal injection (1.5 units) of BM. Control rats received saline. Rats were killed at 3, 5, 7, 14, and 28 days after BM, cytosolic and nuclear proteins were extracted and isolated from lung tissues, and Smad proteins were probed with specific antibodies. In BM-exposed lung tissue, compared with control, Smad3 decreased persistently in the cytosol and increased transiently in the nucleus. There was a persistent increase in phosphorylation and nuclear accumulation of Smad2/3. Smad4 was increased transiently in both the cytosol and nucleus. A significant and progressive decrease in the expression of Smad7, the endogenous inhibitor of TGF-β/Smad signaling, was observed after BM instillation. Collectively, our results indicate that an imbalance between agonistic Smads2–4 and antagonistic Smad7 may result in the unchecked activation of an autocrine TGF-β loop, which contributes to the pathogenesis of BM-induced pulmonary fibrosis.

PULMONARY FIBROSIS IS CHARACTERIZED by inflammation, excessive proliferation of fibroblasts, and abnormal deposition of extracellular matrix (ECM) proteins. Inflammatory cells produce cytokines and growth factors, which modulate fibroblast proliferation and synthesis of ECM proteins (3, 20). Transforming growth factor-β (TGF-β) has been widely implicated in the fibrotic process and has been shown to upregulate the production of several ECM proteins (1). Alveolar macrophages and fibroblasts from fibrotic lungs secrete higher amounts of active TGF-β1 protein compared with normal lungs (14, 29). Overexpression of TGF-β1 induces fibrosis in the rat lung (25); neutralization of TGF-β1 results in marked attenuation of fibrosis (7). Therefore, it has been reasoned that inhibition of TGF-β1 activity will have potential benefits in suppressing fibrotic lung diseases.

Recent advances have enhanced our understanding of the Smad proteins, which are critical components of the TGF-β signaling pathway (24). TGF-β couples to transmembrane serine/threonine kinase receptors and activates intracellular mediators known as Smads (16). Activation of Smads causes their translocation from the cytoplasm to the nucleus, where they function to control gene expression (17). Three major groups of Smads have been identified: the receptor-regulated Smads (R-Smads, Smad2, and Smad3) that, when phosphorylated, associate with another class of Smads, known as the “common” Smad (Co-Smad, Smad4). Co-Smad forms a heterooligomeric complex with the R-Smads. The R-Smad/Co-Smad complex then translocates to the nucleus, where it can bind to TGF-β-responsive promoter DNA, either directly through the Smad-binding elements or in conjunction with other sequence-specific DNA-binding proteins, and regulates gene expression. The inhibitory Smads (Smad6 and Smad7) antagonize signaling by preventing R-Smad phosphorylation and/or by interfering with the R-Smad/Co-Smad complex. Hence, Smad family proteins are critical components of the TGF-β signaling pathway (16).

Although it is well described that TGF-β plays a major role in lung fibrosis and that Smad proteins are important intracellular mediators of TGF-β signaling, relatively little is known about the specific molecular changes underlying exaggerated TGF-β1 activity in fibrotic lung disease. Changes in Smad protein expression have been implicated in various human diseases, including cancer (21), kidney and liver fibrosis (6, 15), and autoimmune diseases such as scleroderma (5). In terms of lung fibrosis, Zhao and Geverd (32) reported alterations in Smad3 expression in bleomycin (BM)-treated rats, suggesting the involvement of Smad proteins in this disease process. In two separate studies, transient gene transfer and overexpression of Smad7 (19) and deficiency of Smad3 attenuated BM-induced lung fibrosis in mice (31). A detailed description of changes in the various Smad proteins and in the kinetics of these changes in response to BM is lacking. In addition, the endogenous expression of Smads in normal lung has not been well described. Therefore, in the current study, we investigated the expression of Smads in normal lungs and the temporal modulation of different members of the Smad family (Smad2, Smad3, Smad4, and Smad7) in response to an administration of BM known to provoke pulmonary fibrosis. Furthermore, we defined changes in the subcellular distribution of these molecules.

MATERIALS AND METHODS

Chemicals. Reagents were obtained from the following sources: BM (Blenoxane) was from Bristol-Myers Squibb (Princeton, NJ);
EDTA, N-ethylmaleimide, N,N'N'-methylene-bisacrylamide, N,N',N',N'-tetra methylenediamine, Nuclei EZ Prep Nuclear Isolation kit, PMSF, protease and phosphatase inhibitor cocktails, Triton X-100, Trizma base, Tween 20, β-actin, and vimentin antibody were from Sigma (Oakville, ON, Canada); hyperfilm, Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane, molecular weight standards, and ECL Western blotting detection reagents were from Amersham Pharmacia Biotech (Montreal, QC, Canada); primary antibodies against phosphorylated Smad2/3, Smad3, Smad4, and Smad7 and horseradish peroxidase-labeled anti-goat secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); fluorescent secondary antibodies for Smad4 (Alexa Fluor 546) and vimentin (Alexa Fluor 488) were obtained from Molecular Probes (Burlington, ON, Canada). All other reagents were of analytical grade and commercially available.

**Experimental design.** All animal experiments were carried out following standard ethical procedures and in accordance with institutional guidelines (McGill Univ., Montreal, Canada). Pulmonary fibrosis was induced in male Sprague-Dawley rats by a single intratracheal instillation of BM as previously described (28). One-week-old rats were anesthetized with pentobarbital sodium (30 mg/kg ip) and intubated with a tracheal cannula. BM (1.5 units) was dissolved in 0.3 ml of sterile physiological saline and instilled into the rat’s lungs via the tracheal cannula. Control rats received the same volume of saline only. Both saline- and BM-treated rats were monitored daily, and body weights were recorded. At 3, 5, 7, 14, and 28 days after BM instillation, rats were anesthetized with pentobarbital sodium (60 mg/kg ip), the thorax was opened, and animals were killed by severing the inferior vena cava. The lungs were then excised. One lung was fixed with Histocon, covered with optimum cutting temperature compound, frozen while inflated, and stored at −80°C. The other lung was processed for isolation and characterization of Smad proteins by Western blot analysis. Four rats were studied in each group.

**Extraction of lung cytosolic and nuclear proteins.** The preparation of cytosol and nuclear extracts was based on the method of Hao et al. (10) and Isono et al. (13) with minor modifications. Lung tissues from saline- and BM-treated rats were homogenized in 0.1 M Tris (pH 7.4) containing proteinase and phosphatase inhibitor cocktails and 0.1% Triton X-100. The homogenates were sonicated for 5 s (repeated 5 times) to disrupt the nuclear membranes. The samples were allowed to lyse for 15 min on ice, and after centrifugation at 10,000 g for 20 min at 4°C, the supernatant was assayed for the cytosolic Smad protein. The pellet was resuspended in 0.1 M Tris (pH 7.4) containing 0.001 M EDTA and proteinase and phosphatase inhibitor cocktails. Samples were then sonicated for 15 s (repeated 4 times) to disrupt the nuclei and allowed to lyse for 20 min on ice. After centrifugation at 10,000 g for 20 min at 4°C, the supernatant was assayed for the nuclear Smad protein. Protein concentrations were determined by Bio-Rad protein assay.

**SDS-PAGE and Western blot analysis of Smad proteins.** Specific goat polyclonal antibodies were used to identify the expression of Smad proteins. Equal amounts (50 µg) of proteins from cytosolic or nuclear fractions were resolved on a 10% SDS-PAGE. After electrophoresis, the separated proteins were electrophoretically transferred to nitrocellulose membranes and blocked for 1 h at room temperature in Tris-buffered saline with 0.2% Tween 20 (TBST) containing 5% dry milk. After being blocked, membranes were washed with TBST and then incubated with primary antibodies (Smads 3, 4, 7 and phosphorylated Smad2/3, goat polyclonal) at a dilution of 1:1,000 for 1 h at room temperature. After being washed with TBST, membranes were incubated with a 1:1,000 dilution of horseradish peroxidase-labeled rabbit anti-goat secondary antibody for 1 h at room temperature. After further washing with TBST, membranes were visualized by ECL. In preliminary experiments, to confirm equal amounts of protein loading, the membrane was stripped and reprobed with β-actin antibody. There was no alteration in β-actin protein expression with BM exposure (data not shown).

**Quantification of Western blots.** Densitometric analysis of Smad proteins was performed with image analyzer software (Fluorchem; Alpha Innotech, San Leandro, CA) that measures the sum of all the pixel values after background correction. The mean values of four observations are presented, and values were expressed in arbitrary densitometric units.

**Immunofluorescent localization of Smad4 and vimentin.** Immunofluorescent staining was used to identify the cells expressing the Smad proteins. Lung tissues were cut into 5-µm sections with a cryostat. Tissue sections were fixed with ice-cold acetone-methanol and then dried. The sections were blocked by incubating with universal blocking solution for 1 h, followed by incubation with 10% normal goat serum. Slides were then rinsed with TBS and incubated overnight with primary antibodies for Smad4 (goat polyclonal) and vimentin (mouse monoclonal) at a dilution of 1:500 at 4°C. After being washed with TBS, sections were incubated with fluorescein-labeled secondary antibodies (rabbit, anti-goat for Smad4, and goat, anti-mouse for vimentin) at a dilution of 1:1,000 for 1 h at room temperature. After being washed in TBS, stained sections were sealed with crystal mount and viewed with an Olympus fluorescence microscope.

**Statistical analysis.** All values are reported as means ± SE of four observations. One-way ANOVA with Newman–Keuls post hoc correction was used to compare the differences among multiple groups (GraphPad Prism, version 3.0).

**RESULTS**

Histological examination of BM lungs revealed infiltration of inflammatory cells in the alveolar space and the interstitium and focal fibrotic lesions, as reported previously (28).

**Smad expression in normal rat lungs.** Healthy lung tissues constitutively expressed the following Smad proteins: Smad3 and Smad4 in the cytosol and nucleus; phosphorylated Smad2 and Smad3 in the nucleus; and Smad7 in the cytosol (Figs. 1–6).

**Decreased Smad7 expression in fibrotic lungs.** The TGF-β/β-Smad signaling system is well known for an autoinhibitory loop that involves Smad7; therefore, the expression level of Smad7 influences TGF-β transcriptional responsiveness. Detection of the Smad7 protein was investigated using Western blot analysis of lung tissue obtained from rats with BM-induced fibrosis. A 43-kDa band corresponding to the Smad7 protein was observed with decreased intensity 3 days after BM instillation compared with control tissues (Fig. 1). This decrease persisted in a time-dependent fashion over 28 days. There was a 28, 42, 51, 59, and 71% decrease in the levels of Smad7 protein after 3, 5, 7, 14, and 28 days of BM instillation, respectively. These findings suggest that decreased Smad7 expression may be an important mechanism underlying enhanced TGF-β activity in fibrotic lungs.

**Increased Smad3 expression in fibrotic lungs.** Compared with normal lungs, there was a significant decrease in cytosolic Smad3 expression in fibrotic lungs at all time points following BM administration (Fig. 2). To determine whether translocation of Smad3 to the nucleus explained the decline in cytosolic Smad3, nuclear proteins were probed with Smad3 antibody. Densitometric analysis revealed increased nuclear accumulation of Smad3 protein at days 3, 5, and 14 after BM instillation (Fig. 3), with a maximum increase of approximately twofold on day 5 after BM treatment. The decline in cytosolic Smad3, however, persisted even at 28 days.

**Upregulation of phosphorylated Smad2/3 protein in fibrotic lungs.** Because phosphorylation of Smad2/3 by the activated TGF-β1 receptor I is a major step in the initiation of TGF-β1 signal transduction, nuclear proteins extracted from lung tissues were probed with anti-phosphorylated Smad2/3 anti-
body that selectively recognizes phosphorylated Smad2 and Smad3. Whereas normal lung tissues had appreciable nuclear accumulation of phosphorylated Smad2/3 protein, phosphorylation and nuclear accumulation of Smad2/3 were significantly increased in fibrotic lungs (Fig. 4). Nuclear accumulation of phosphorylated Smad2/3 in the fibrotic lung peaked at day 7 (3-fold increase compared with control lungs, \( P < 0.001 \)) and persisted until day 28 (1.7-fold increase compared with controls, \( P < 0.05 \)) after BM instillation (Fig. 4).  

Increased Smad4 expression in fibrotic lungs. Once activated, Smad3 associates with Smad4 and translocates to the nucleus. To study whether increased Smad3 was associated with elevated levels of Smad4, both the cytosolic and nuclear proteins were probed with a Smad4 antibody. Immunoblot analysis displayed a time-dependent increase in the amount of Smad4 in cytosolic fractions in fibrotic lungs relative to normal lungs. Figure 5 indicates that BM instillation resulted in a 1.7-, 2.1-, and 1.9-fold increase of Smad4 protein levels in cytosolic fractions on days 3, 5, and 7, respectively. However, this increase declined toward control levels after day 7 and remained constant through 28 days. Next, we examined Smad4 expression level in nuclear fractions. A similar time-dependent increase in nuclear accumulation of Smad4 protein was detected in fibrotic lungs (Fig. 6).  

Immunolocalization of Smad protein. Immunofluorescent staining revealed colocalization of Smad4 and vimentin (a characteristic fibroblast marker; Fig. 7, A–C).

DISCUSSION

Pulmonary fibrosis is a significant cause of morbidity and mortality. However, the molecular mechanisms that underlie the evolution of the fibrotic process have not been studied in detail. Administration of BM produces inflammation and fibrosis of the...
lung in humans and experimental animals. Using the BM model of pulmonary fibrosis in the rat, we showed enhanced levels of collagen and proteoglycans (PGs) in fibrotic lungs (4, 28). We then examined changes in PG metabolism in an in vitro system using fibroblasts isolated from BM-exposed rats. These cells demonstrated increased production of TGF-β and all subclasses of PGs (29). Further studies demonstrated that exogenous administration of TGF-β enhanced PG expression, whereas anti-TGF-β antibody inhibited it, implicating TGF-β as an important mediator in BM-induced pulmonary fibrosis and matrix production (29).

In the current study, we investigated the temporal changes in agonistic (Smad2, Smad3, and Smad4) and antagonistic (Smad7) Smad proteins during the early and late stages of BM-induced pulmonary fibrosis in rats to identify the molecular defects underlying the increased TGF-β activity that occurs in fibrotic lungs. In vitro studies have shown that the transient induction of Smad7 in response to exogenous TGF-β1 acts as an important negative feedback inhibitor of TGF-β signaling by blocking the activation of Smad2 and Smad3 and preventing their interaction with activated TGF-β receptors and subsequent phosphorylation. In addition, Smad7 can form a complex with R-Smads, preventing their movement into the cell nucleus (11, 18). On the basis of in vitro data, cells with high levels of Smad7 are resistant to the fibrotic actions of TGF-β1, whereas cells with low levels of Smad7 are more vulnerable to this effect. In this regard, we found that BM-induced lung fibrosis is associated with decreased lung levels of Smad7. BM-induced decreases in Smad7 will likely undermine the homeostatic role of Smad7 in modulating TGF-β responses. Evidence consistent with a causal relationship between decreased Smad7 and skin lesions has recently been reported in scleroderma patients (5) and in a murine model of scleroderma (26). Studies have also shown that decreased Smad7 expression contributed to myocardial fibrosis in infarced heart (30) and in Thy-1 nephritis in rats (27). Antisense inhibition of endogenous Smad7 function in normal fibroblasts resulted in enhanced collagen synthesis (2). These observations strengthen the emerging paradigm that Smad7 is an important intracellular antagonist of TGF-β signaling. Nakao et al. (19) demonstrated that transient gene transfer and expression of Smad7 prevented BM-induced pulmonary fibrosis in mice. Protection conferred by Smad7 transgene against BM-induced lung injury in mice ameliorated lung fibrotic lesions and suppressed type I procollagen mRNA and hydroxyproline content. Together, our results suggest that decreased Smad7 expression in the fibrotic lung, resulting in sustained activation of TGF-β/Smad signaling in lesional tissues, could be a critical event in BM-induced lung fibrosis.

There is growing evidence that Smad3 plays a role in the development of pathologic fibrosis. For example, it was demonstrated in a mouse model of CCl4 hepatic fibrosis that induction of collagen synthesis was defective in hepatocytes of Smad3-null mice (23). In addition, lack of Smad3 attenuated BM-induced pulmonary fibrosis in mice (31). Furthermore, Smad3 has been shown to play a role in the transcriptional activation of TGF-β-mediated induction of collagen expression (23). Consistent with these findings, the present study demonstrates that expression of Smad3 protein was altered during inflammation and lung fibro-

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Fig. 4. Increased nuclear accumulation and phosphorylation of Smad2/3 protein in fibrotic lungs. Levels of phosphorylated (p)-Smad2/3 protein were examined in nuclear extracts by Western blot analysis. Densitometric analysis shows increased nuclear accumulation of phosphorylated Smad2/3 in the fibrotic lung peaked at day 7 and persisted until day 28 after bleomycin instillation. Values are means ± SE of 4 animals. One-way ANOVA with post hoc Newman-Keuls test was used for statistical analysis of the data. *, **, ***P < 0.05, 0.01, and 0.001, respectively, vs. control.
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Fig. 5. Increased cytosolic Smad4 expression in fibrotic lungs. Western blotting of Smad4 in cytosolic extracts shows a 2-fold increase of lung Smad4 protein levels on days 3, 5, and 7 after bleomycin instillation. Values are means ± SE of 4 animals. One-way ANOVA with post hoc Newman-Keuls test was used for statistical analysis of the data. **P < 0.001 vs. control.
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Compared with normal lungs, there was a significant reduction of Smad3 in the cytosolic fraction in fibrotic lungs at all time points following BM administration. We reasoned that the decreased expression of Smad3 was due to translocation of this molecule from the cytosol to the nucleus. Cytosol to nuclear translocation of Smad3 in lung fibroblasts exposed to TGF-β has been demonstrated by Zhao and Geverd (32). Indeed, in the current study, Smad3 expression was increased in the nuclear fraction in fibrotic lungs. These observations imply that nuclear translocation of Smad3 is an important step in signal transduction, and phosphorylation and/or activation of Smad3 may be important for its nuclear translocation. Our findings are consistent with the recent work of Takagawa et al. (26), who found increased Smad3 expression in a murine model of BM-induced scleroderma. Smad3 expression is also increased in scleroderma skin and in explanted scleroderma fibroblasts in culture (5). Other reports have demonstrated increased Smad3 expression in postinfarction myocardial fibrosis (9) and during allergen-induced airway inflammation (22). These data, however, are contradicted by the findings of Zhao and Geverd (32), who showed that mRNA signal for Smad3 was decreased in rat lung exposed to BM and in lung fibroblasts incubated with TGF-β.

It has been observed that the signaling activity of Smad2 and Smad3 is modulated through phosphorylation and cytosol-nucleus translocation. In this study, temporal changes in the phosphorylation and nuclear accumulation of Smad2/3 in fibrotic lungs were investigated. A significant increase in phosphorylation and nuclear accumulation of Smad2/3 was observed at all time points. Increases in protein levels were evident as early as 3 days and remained significantly elevated over the control, even at 28 days post-BM treatment. These observations suggest that the prolonged activation of Smad2/3 and its nuclear translocation are important for initiation of TGF-β signals in BM-induced fibrosis, which may be attributed to decreased Smad7 expression in fibrotic lungs. Our findings are consistent with other studies reporting increased phosphorylation of R-Smads in systemic sclerosis (5, 26).

Translocation of Smads into the nucleus and subsequent transcriptional activation require heterooligomer formation of ligand-specific Smads with the common mediator Smad4 after ligand stimulation and phosphorylation of ligand-specific Smads by the receptors. Once activated, Smad3 associates with Smad4 and translocates to the nucleus (16). To investigate whether upregulation and nuclear translocation of Smad3 were associated with increased expression of Smad4, nuclear and cytosolic proteins were probed with a Smad4 antibody. Our results show that Smad4 levels were induced during the early inflammatory and remodeling phase of BM-induced lung injury. These observations suggest that increased expression and nuclear accumulation of Smad3 and Smad4 may play an important role in the induction of ECM genes and thus contribute to the ongoing remodeling of lung tissue during fibrogenesis. Consistent with our observation, increased Smad4 expression and nuclear accumulation have been shown in myocardial infarction (9) and in hepatic cells from fibrotic liver (1). Increased Smad4 levels were also reported in allergen-
challenged mice (22). Elevated cytoplasmic Smad4 protein has also been shown to accelerate TGF-β1 signaling in renal tubulointerstitial cells of hereditary nephrotic mice with chronic renal fibrosis (8).

Because inflammation, mesenchymal cell proliferation, and fibroblast activation characterize pulmonary fibrosis, we were interested to determine the cell type responsible for altered Smad expression in fibrotic lungs. Immunofluorescent staining revealed that Smad4 expression was localized to cells expressing vimentin (Fig. 7). These results identify fibroblasts as a cellular source of Smad protein in the fibrotic tissue and support our earlier findings, which demonstrated increased production of TGF-β1 and PGs by BM lung fibroblasts (32).

In conclusion, our findings indicate that alterations in the expression of various Smad protein components occur during the development of BM-induced pulmonary fibrosis in rats. Cytosol-to-nucleus translocation of Smad3 and nuclear accumulation and phosphorylation of Smad2/3 were significantly increased during the development of BM-induced pulmonary fibrosis. These changes were paralleled by a significant decrease in Smad7 expression in fibrotic lungs. Because Smad7 plays a key role in the negative feedback loop of TGF-β1 signal transduction, we believe that a decrease in Smad7 expression contributes to the increased TGF-β1 signaling observed in BM-induced lung fibrosis.

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GRANTS

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