Transforming Growth Factor (TGF)-β₁ Stimulates Pulmonary Fibrosis and Inflammation via a Bax-dependent, Bid-activated Pathway That Involves Matrix Metalloproteinase-12*§

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Hye-Ryun Kang†, Soo Jung Cho†, Chun Geun Lee†, Robert J. Homer§, and Jack A. Elias††

From the ††Section of Pulmonary and Critical Care Medicine and the §Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06520

Fibrosis, apoptosis, and the exaggerated production of transforming growth factor (TGF)-β₁ are juxtaposed in a variety of pulmonary diseases including the interstitial lung diseases and asthma. In these disorders, the relationships between these responses are not well defined. In addition, the apoptosis pathways that contribute to these responses and the mechanism(s) of their contribution have not been described. We hypothesized that BH3 domain-only protein-induced apoptosis plays an important role in the pathogenesis of TGF-β₁-induced pulmonary responses. To test this hypothesis, we characterized the effects of transgenic TGF-β₁ in mice with wild type (WT) and null Bax loci. To investigate the mechanisms of Bax activation and its effector functions, we also compared the effects of TGF-β₁ in mice with WT and null Bid and matrix metalloproteinase (MMP)-12 loci, respectively. These studies demonstrate that TGF-β₁ is a potent stimulator of Bax, Bid, and MMP-12. The studies also demonstrate that Bax and Bid play key roles in the pathogenesis of TGF-β₁-induced inflammation, fibrosis, and apoptosis; that TGF-β₁ stimulates MMP-12, TIMP-1, and cathepsins and inhibits MMP-9 and p21 via Bax- and Bid-dependent mechanisms; and that TGF-β₁-stimulated pulmonary fibrosis is ameliorated in MMP-12-deficient animals. Finally, they demonstrate that Bax, Bid, and MMP-12 play similar roles in bleomycin-induced fibrosis, thereby highlighting the importance of this Bid-activated, Bax-mediated pathway and downstream MMP-12 in a variety of fibrogenic settings.

Fibrosis is an important cause of morbidity and mortality in the lung and other organs. This can be seen in the interstitial lung diseases (ILD) including idiopathic pulmonary fibrosis (IPF), scleroderma, radiation-induced pulmonary fibrosis, and bleomycin lung where matrix molecule deposition, enhanced collagen accumulation, and alveolar septal rupture with honey-combing are seen and can lead to fatal consequences (1–4). It is also seen in asthma, which is characterized by chronic inflammation and subepithelial airway fibrosis (5, 6). Pathologic examinations have highlighted the frequent juxtaposition of fibrosis, structural cell (usually epithelial cell) apoptosis, and the exaggerated production of transforming growth factor (TGF)²-β₁ in these diseases and models of these disorders (1, 7–22). Studies have also demonstrated that TGF-β₁ plays an essential role in wound healing and matrix molecule deposition, and induces fibrotic and alveolar remodeling responses in vivo (14, 16, 17, 20–23). They also demonstrated that TGF-β₁ is a potent stimulator of epithelial apoptosis (22, 24–27) and that interventions that block apoptosis can ameliorate fibrotic and alveolar remodeling responses in a variety of experimental systems (7, 22). As a result of these studies, TGF-β₁ is believed to be a critical mediator of pathologic, fibrotic, and remodeling responses in the lung and other organs, and these responses are believed to be mediated by the ability of TGF-β₁ to activate fibrogenic and apoptotic “injury” pathways. The components of these pathways, however, are poorly understood. In particular, the apoptosis responses that contribute to TGF-β₁-induced tissue fibrosis and alveolar remodeling have not been defined, and the mechanisms via which apoptosis contributes to these fibrogenic and remodeling responses have not been adequately investigated.

Although cell death can be triggered by a vast array of stimuli and mediated via an increasingly complex series of pathways, the vast majority of signals engage the cell death machinery at the level of the cell membrane and/or at the level of the mitochondria. The membrane (“extrinsic”) pathway triggers surface “death receptors” such as Fas, which binds Fas ligand and TNF receptor 1, which binds TNF and lymphotoxin (9, 10, 28). Other stimuli use mitochondrial dysfunction to signal death responses. In this “intrinsic” response, the balance between pro-apoptotic and anti-apoptotic B cell lymphoma (Bcl)-2 family members is altered with BH3 domain-only family members

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

‡ To whom correspondence should be addressed: Section of Pulmonary and Critical Care Medicine, Yale University School of Medicine, New Haven, CT 06520-8057. Tel.: 203-737-4249; Fax: 203-785-3826; E-mail: jack.elias@yale.edu.

§ The abbreviations used are: TGF, transforming growth factor; MMP, matrix metalloproteinase; WT, wild type; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; ELISA, enzyme-linked immunosorbent assay; Tg, transgenic.
like Bid being activated, neutralizing anti-apoptotic Bcl-2 proteins and binding to pro-apoptotic molecules like Bax. These interactions allow Bax to form or interact with mitochondrial pores, release a variety of mediators including cytochrome c and Smac/DIABLO, activate caspase-9, and induce cell death (9, 10, 28–34). Studies from our laboratory and others have demonstrated that TGF-β1 is a potent stimulator of a variety of cell death pathways in the lung and other organs (20, 22, 25–27). They also demonstrated that this apoptosis is a critical prerequisite for TGF-β1-induced fibrosis and alveolar remodeling (22). The relative contributions that the different cell death pathways make to these responses, however, have not been investigated. In addition, the mechanisms by which these pathways contribute to tissue fibrosis have not been adequately defined.

We hypothesized that BH3 domain-only protein-induced mitochondrial dysfunction plays an important role in the pathogenesis of TGF-β1-induced pulmonary fibrosis. To test this hypothesis, we characterized the effects of transgenic TGF-β1 in mice with wild-type and null mutant Bax loci. To investigate the mechanisms of Bax activation and its effector functions, we also compared the effects of transgenic TGF-β1 in mice with wild-type and null Bid and mice with wild-type and null matrix metalloproteinase (MMP)-12 loci, respectively. These studies demonstrate that TGF-β1 is a potent stimulator of Bax, Bid, and MMP-12 in the murine lung. They also demonstrate that Bax and Bid play key roles in the pathogenesis of TGF-β1-induced inflammation and fibrosis and bleomycin-induced fibrosis and that TGF-β1 regulates the expression of MMPs, anti-proteases and cathepsins, but not collagens via a Bax- and Bid-dependent mechanism(s). Finally, they demonstrate that MMP-12 also plays an important role in TGF-β1 effector pathway activation, because TGF-β1-stimulated and bleomycin-stimulated pulmonary fibrosis were ameliorated in MMP-12-deficient animals.

**EXPERIMENTAL PROCEDURES**

**Overexpression Transgenic and Null Mutant Mice**—CC10-tTS-rTA-TGF-β1 transgenic (Tg) mice were generated in our laboratory, bred onto a C57BL/6 background for >10 generations, and used in these studies. These mice utilize the Clara cell 10-kDa protein (CC10) promoter to specifically target TGF-β1 to the lung. The methods that were used to generate and characterize these mice have been described previously (22). Mice with null mutations of Bax or Bid that had been bred onto a C57BL/6 background for >10 generations were generated in the laboratory of the late Dr. Stanley Korsmeyer as previously described (34, 61). The Bax-null mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The Bid-null mice were a generous gift from Dr. Korsmeyer. MMP-12-null mice were a generous gift from Dr. Steven Shapiro (University of Pittsburgh) (62). These mice were bred with the TGF-β1 Tg mice to obtain Tg mice with WT and null loci.

**Doxycycline Water Administration**—Six-week-old transgene (+) mice and transgene (−) littermate controls were randomized to normal water or water containing 0.5 mg/ml of doxycycline as described previously (22). Phenotypic alterations were evaluated at intervals thereafter.

**Quantification of Lung Collagen**—Animals were anesthetized, a median sternotomy was performed, and right heart perfusion was accomplished with calcium- and magnesium-free phosphate-buffered saline. The heart and lungs were then removed en bloc. The right lung was frozen in liquid nitrogen and stored at −80 °C until used. The collagen content was determined by quantifying total soluble collagen using the Sircol Collagen Assay kit (Biocolor) according to the manufacturer’s instructions. The data are expressed as the collagen content of the entire right lung.

**Histologic Analysis**—The lungs were removed en bloc as described above, inflated at 25-cm pressure with phosphate-buffered saline containing 0.5% low melting point agarose gel, fixed, embedded in paraffin, sectioned, and stained. Hematoxylin and eosin, and Mallory’s trichrome stains were performed in the Research Histology Laboratory of the Department of Pathology at the Yale University School of Medicine.

**Morphometric Analysis**—Alveolar remodeling was estimated from the mean chord length of the airspace as described previously by our laboratory (22, 35).

**BAL and Lung Inflammation**—Lung inflammation was assessed by BAL as described previously (22). The BAL samples from each animal were pooled and centrifuged. The number and type of cells in the cell pellet were determined with light microscopy.

**TdT-mediated dUTP Nick End-labeling (TUNEL) Evaluations**—End-labeling of exposed 3′-OH ends of DNA fragments was undertaken with the TUNEL in situ cell death detection kit AP (Roche Applied Science) as described by the manufacturer. After staining, 20 fields of alveoli were randomly chosen for examination. The labeled cells were expressed as a percentage of total nuclei.

**mRNA Analysis**—mRNA levels were assessed using real time reverse transcription PCR assays described by our laboratories (22, 63). In these assays, total cellular RNA from lungs were obtained using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The primer sequences that were employed and have not been previously reported by our laboratory can be found in supplemental Table S1.

**Quantification of TGF-β1**—The levels of BAL TGF-β1 were determined by ELISA (R&D Systems, Inc., Minneapolis, MN) per the manufacturer’s instructions. These evaluations were done before and after acid activation to assess the levels of activated and total TGF-β1, respectively.

**Immunoblot Analysis**—Lung lysates were prepared, and Western analysis was undertaken with antibodies that reacted selectively with ICAD (Chemicon International, Temecula, CA) or MMP-12 (Santa Cruz Biotechnology) as described previously (22, 64).

**Bleomycin Administration**—Bleomycin (0.075 units/mouse) or vehicle control were administered to C57BL/6 female mice as previously described by Liang et al. (65).

**Statistics**—Normally distributed data are expressed as means ± S.E. and assessed for significance by Student’s t test or analysis of variance as appropriate. Data that were not normally distributed were assessed for significance using the Wilcoxon rank sum test.
RESULTS

TGF-β1 Regulation of Bax—To address the possibility that intrinsic cell death pathways play important roles in the pathogenesis of TGF-β1-induced tissue responses, studies were first undertaken to determine if the expression of Bax was regulated by transgenic TGF-β1. This was done by comparing the levels of Bax mRNA and protein in lungs from doxycycline-treated Tg mice (Fig. 1). This induction was seen after as little as 24 h of transgene activation. These studies demonstrate that TGF-β1 is a potent stimulator of Bax mRNA and protein accumulation in lungs from doxycycline-treated Tg mice (Fig. 1).

FIGURE 1. TGF-β1 regulation of Bax mRNA and protein. WT and Tg mice were incubated with doxycycline for 2 weeks. Real time RT-PCR (A) and Western analyses (B) were used to compare the levels of Bax mRNA and protein. The values in A represent the mean ± S.E. of evaluations in a minimum of five mice (*, p < 0.05). B is representative of at least three similar experiments and shows Bax expression in the lung tissues from 1 wild type and 3 transgenic animals.

FIGURE 2. Roles of Bax in TGF-β1-mediated alveolar remodeling. Tg(−/−) and Tg(+/+) mice with WT(+/+) and null(−/−) Bax loci were generated and evaluated after 2 weeks of transgene activation. Total BAL cell recovery was quantitated (A), and fibrosis was evaluated with histologic trichrome stains (B) and assays of collagen content (Sircol) (C). The values represent the mean ± S.E. of evaluations in a minimum of five mice (**, p < 0.01; NS, not significant at p = 0.05).

FIGURE 3. Roles of Bax in TGF-β1-induced apoptosis. Tg(−/−) and Tg(+/+) mice with WT(+/+) and null(−/−) Bax loci were generated and evaluated after 48 h of transgene activation. The percentage of cells that were TUNEL(+) was quantitated (A) and the levels of cleaved ICAD were evaluated with Western evaluations (B). The values in A represent the mean ± S.E. of evaluations in a minimum of five mice (*, p < 0.01; **, p < 0.001).

FIGURE 4. Roles of Bax in TGF-β1-mediated inflammation and fibrosis. Tg(−/−) and Tg(+/+) mice with WT(+/+) and null(−/−) Bax loci were generated and evaluated after 4 weeks of transgene activation. Total BAL cell recovery was quantitated (A), and fibrosis was evaluated with histologic trichrome stains (B) and assays of collagen content (Sircol) (C). The values represent the mean ± S.E. of evaluations in a minimum of five mice (*, p < 0.05; **, p < 0.01).
3 days of doxycycline administration and persisted throughout the 28-day study interval (Fig. 1 and data not shown).

Roles of Bax in TGF-β1-induced Inflammation and Fibrosis—To determine if the inflammatory and fibrotic effects of TGF-β1 were altered in the absence of Bax, we used BAL, histologic, and biochemical (Sircol) approaches to quantify the inflammation and collagen in lungs from Tg mice with WT and null Bax loci. In accord with previous studies from our laboratory (22), transgenic TGF-β1 caused a mononuclear cell-predominant inflammatory response and a significant increase in lung collagen content in mice that expressed Bax normally (p < 0.001) (Fig. 2, A–C). Interestingly, both responses were diminished in the absence of Bax. After 14 days of doxycycline water administration, TGF-β1-induced BAL cellularity was decreased by 60.1 ± 5.5% (p = 0.044) in Bax-null versus WT animals (Fig. 2A). BAL and tissue differential cell counts, however, were not significantly altered (data not shown). Similarly, at this time point, TGF-β1-induced collagen accumulation was decreased by 80.3 ± 6.1% in Tg(+) Bax-null versus WT mice (p = 0.015) (Fig. 2C). These studies demonstrate that Bax plays an important role in the pathogenesis of TGF-β1-induced inflammation and fibrosis in the murine lung.

Role of Bax in Alveolar Remodeling—In addition to inducing tissue fibrosis, TGF-β1 induces alveolar remodeling with septal destruction and an increase in alveolar chord length (22). To define the role(s) of Bax in these responses, we compared the alveoli from Tg mice with WT and null Bax loci. In accord with our prior report (22), an increase in lung destruction was readily apparent in Tg mice that produced Bax normally (Fig. 3). Interestingly, null mutations of Bax did not significantly alter this alveolar remodeling (Fig. 3). Thus, although Bax plays an important role in TGF-β1-induced inflammation and fibrosis, it does not contribute, in a major way, to TGF-β1-induced alveolar remodeling.

Role of Bax in TGF-β1-induced DNA Injury and Cell Death—Previous studies from our laboratory demonstrated that TGF-β1 activates a number of apoptosis pathways in the murine lung (22). Thus, studies were undertaken to define the contribution of Bax-induced mitochondrial alterations in TGF-β1-induced apoptosis. This was done using TUNEL stains to compare the TGF-β1-induced DNA injury and cell death in transgenic mice with WT and null Bax loci. Transgenic TGF-β1 caused an impressive increase in TUNEL staining in mice with WT Bax loci (Fig. 4A). These TUNEL(+) cells were largely epithelial cells as evidenced by their histologic location and morphology (data not shown). This response was readily appreciated after 2 days of doxycycline administration and decreased with longer periods of Tg activation (Fig. 4A and data not shown). Bax appeared to play an important role in this response because the TUNEL staining was decreased in lungs from Tg(+) mice with null mutations of Bax (Fig. 4A). This was readily appreciated after 48 h of doxycycline administration where TGF-β1-induced TUNEL staining was decreased by 52.2 ± 4.1% compared with Tg mice with

FIGURE 5. Roles of Bax in TGF-β1-regulation of Egr-1, cathepsins, and p21. Tg(+) and Tg(−) mice with WT(+/+) and null(−/−) Bax loci were generated and evaluated after 2 weeks of transgene activation. The levels of mRNA encoding the noted cathepsins (Cat-S) was evaluated by real time-RT-PCR (A), and the levels of BAL cathepsins were evaluated via Western analysis (B). Real time-RT-PCR was also used to quantitate p21 mRNA (C). The values in A and C represent the mean ± S.E. of evaluations in a minimum of five mice. B is representative of at least four similar experiments (*, p < 0.05; **, p < 0.01).
WT Bax loci (Fig. 4A) \( p = 0.028 \). This inhibition was associated with a significant decrease in the accumulation of activated caspase-3 and a decrease caspase-mediated ICAD (inhibitor of caspase-activated DNase) cleavage (Fig. 4B and data not shown). Thus, these studies demonstrate that Bax-mediated mitochondrial alterations are important contributors to TGF-\( \beta_1 \)-induced DNA injury and cell death in the murine lung. They also highlight a Bax-independent cell death pathway that is induced by TGF-\( \beta_1 \) in the murine lung.

**Bax and the Regulation of Transgenic TGF-\( \beta_1 \)**—The decreased ability of TGF-\( \beta_1 \) to induce tissue responses in the absence of Bax could be caused by a decrease in the production of transgenic TGF-\( \beta_1 \) or a decrease in its ability to activate its effector pathways. To differentiate among these options, we compared the levels of total and active TGF-\( \beta_1 \) in lungs from Tg(-) and Tg(+) mice with WT and null Bax loci. These effects appeared to be due, in great extent, to alteration(s) in TGF-\( \beta_1 \) effector pathway activation because the levels of total and bioactive TGF-\( \beta_1 \) in BAL from Tg(+) mice with null Bax loci were comparable or greater than those in fluids from Tg(+) mice that make Bax normally (data not shown).

**Bax and TGF-\( \beta_1 \)-induced Apoptosis**—We demonstrated previously that TGF-\( \beta_1 \) induces lung epithelial cell apoptosis via an Egr-1-dependent mechanism and that cathepsins are important regulators of lung epithelial cell death pathways (22, 35). To gain insight into the mechanisms by which Bax might contribute to TGF-\( \beta_1 \)-induced apoptosis, the ability of TGF-\( \beta_1 \) to regulate Egr-1 and cathepsins in the presence or absence of Bax was evaluated. We also characterized the ability of TGF-\( \beta_1 \) to regulate the apoptosis regulator p21 in these settings. TGF-\( \beta_1 \) was a potent stimulator of the levels of Egr-1, cathepsins -S, -D, -K, -H, and -B and p21 mRNA and/or protein in Tg(+) mice (Fig. 5, A–C). These responses were seen as little as 48 h after doxycycline activation and persisted throughout 2-week study interval (Fig. 5 and data not shown). These responses were mediated, at least in part, by Bax because null mutations of Bax decreased the ability of TGF-\( \beta_1 \) to stimulate Egr-1 and the cathepsins (Fig. 5, A and B). Interestingly, TGF-\( \beta_1 \) stimulation of p21 was enhanced in Bax-null mice (Fig. 5C). When viewed in combination, these studies demonstrate that Bax plays an important role in TGF-\( \beta_1 \) stimulation of Egr-1 and the activation of cathepsin-mediated cell death pathways. They also highlight a Bax-dependent mechanism that feeds back to inhibit p21 levels in TGF-\( \beta_1 \) Tg(+) mice.

**Bax and the Mechanisms of Pulmonary Fibrosis**—To address the mechanisms by which Bax might contribute to the pathogenesis of TGF-\( \beta_1 \)-induced fibrosis, we characterized the regulation of collagens and selected proteases and anti-proteases in lungs from TGF-\( \beta_1 \) Tg mice with WT and null Bax loci. These studies demonstrated that TGF-\( \beta_1 \), a potent stimulator of the accumulation of mRNA encoding \( \alpha_1(II) \), \( \alpha_1(III) \), \( \alpha_2(III) \) collagens, fibronectin (FN), elastin, laminin, and type IV collagen (Fig. 6 and data not shown). TGF-\( \beta_1 \) also stimulated the accumulation of mRNA encoding TIMP-1, TIMP-3, and MMP-12 while inhibiting MMP-9 (Fig. 6). Bax played an
Bax, Bid, and MMP-12 Regulation of TGF-ß Effector Function

**FIGURE 7. Role of Bax in bleomycin-induced pulmonary fibrosis.** Tg(−) mice with WT(+/+) and null(−/−) Bax loci were generated and collagen content was evaluated 3 weeks after bleomycin administration. The values represent the mean ± S.E. of evaluations in a minimum of five mice (*, p < 0.05; **, p < 0.01).

important role in the stimulation of TIMP-1 and MMP-12, because TGF-ß stimulation of TIMP-1 and MMP-12 was significantly decreased in mice with null mutations of Bax (Fig. 6). In contrast, the expression of TIMP-3 and MMP-9 was increased in Tg(+) mice with null mutations of Bax (Fig. 6).

Bax in Bleomycin-induced Pulmonary Fibrosis—Studies were also undertaken to determine if the biology of Bax that was defined using our Tg modeling system was also relevant to fibrotic responses that were induced by other stimuli. Bleomycin was chosen because TGF-ß stimulation of TIMP-1 and MMP-12 was significantly decreased in mice with null mutations of Bax (Fig. 6). In accord with our findings with Bid and Bax, the latter studies demonstrate that TGF-ß-stimulated and bleomycin-stimulated pulmonary fibrosis are significantly ameliorated and TGF-ß1-induced alveolar remodeling was not significantly altered in MMP-12 null animals (Fig. 10, C–E). In contrast to Bid and Bax, MMP-12 did not contribute, in a significant fashion, to TGF-ß1-induced inflammation because BAL and tissue inflammation were not significantly altered in comparisons of Tg(+) mice with WT and null MMP-12 loci (data not shown). When viewed in combination, these studies demonstrate that TGF-ß1 stimulates and activates MMP-12 via Bid- and Bax-dependent mechanisms. They also demonstrate that MMP-12 plays an important role in the contributions of Bax and Bid to the pathogenesis of the tissue effects of TGF-ß1 and bleomycin.

DISCUSSION

To gain insight into the pathogenesis of the wound healing, fibrosis, and inflammatory responses that occur at sites of tissue injury and repair, we tested the hypothesis that mitochondrial pathway-mediated apoptosis plays a critical role in the pathogenesis of the inflammatory and remodeling responses induced by TGF-ß1. This was done by comparing the effects of transgenic TGF-ß1 in wild-type mice and mice with null mutations of the critical BH3 domain-only protein Bax and its activator Bid. Because TGF-ß1 stimulation of MMP-12 was shown to be Bid- and Bax-dependent, we also hypothesized that Bid and Bax mediate their TGF-ß1-relevant effects, at least in part, through MMP-12. To address this hypothesis, we also evaluated the effects of transgenic TGF-ß1 in mice with wild-type and null MMP-12 loci. These studies highlight previously unrecognized relationships between TGF-ß1, and Bax, Bid, and MMP-12. Specifically, they demonstrate that TGF-ß1 is a potent stimulator and/or activator of Bax, Bid, and MMP-12. They also demonstrate that Bax and Bid play critical roles in TGF-ß1-induced inflammation, fibrosis, and apoptosis, but do not contribute to

associated with significant changes in collagen and matrix molecule gene expression and were associated with significant decreases in TGF-ß1-stimulated TIMP-1, MMP-12, and cathepsin -S, -B, -D, -K, and -H gene expression (Fig. 9, A and B and data not shown). They were also not TGF-ß1-specific because a similar decrease in bleomycin-induced fibrosis was seen in Bid-null mice (Fig. 9C). When viewed in combination, these studies demonstrate that Bid and Bax play similar roles in the pathogenesis of TGF-ß1 and bleomycin-induced responses in the murine lung. This suggests that Bid activation and Bid-Bax interactions are important contributors to the pathogenesis of TGF-ß1-induced tissue inflammatory and fibrotic responses.

Role(s) of MMP-12—The studies noted above demonstrate that TGF-ß1 stimulates MMP-12 gene expression via Bid- and Bax-dependent mechanisms. To gain additional insight into the contributions of this TGF-ß1-MMP-12 pathway, the effects of transgenic TGF-ß1 and bleomycin on MMP-12 production were evaluated, and the tissue effects of transgenic TGF-ß1 and bleomycin in mice with WT and null MMP-12 loci were evaluated. The former studies demonstrate that TGF-ß1 and bleomycin are potent stimulators of the propeptide and mature forms of MMP-12 (Fig. 10, A and B). In accord with our findings with Bid and Bax, the latter studies demonstrate that TGF-ß1-stimulated and bleomycin-stimulated pulmonary fibrosis are significantly ameliorated and TGF-ß1-induced alveolar remodeling was not significantly altered in MMP-12 null animals (Fig. 10, C–E). In contrast to Bid and Bax, MMP-12 did not contribute, in a significant fashion, to TGF-ß1-induced inflammation because BAL and tissue inflammation were not significantly altered in comparisons of Tg(+) mice with WT and null MMP-12 loci (data not shown). When viewed in combination, these studies demonstrate that TGF-ß1 stimulates and activates MMP-12 via Bid- and Bax-dependent mechanisms. They also demonstrate that MMP-12 plays an important role in the contributions of Bax and Bid to the pathogenesis of the tissue effects of TGF-ß1 and bleomycin.
The pathogenesis of TGF-\(\beta_1\)-induced alveolar remodeling. Null mutations of MMP-12 also ameliorated TGF-\(\beta_1\)-induced fibrosis. They did not, however, alter TGF-\(\beta_1\)-induced inflammation or remodeling. Mechanistic insights were also obtained from studies that demonstrated that TGF-\(\beta_1\) regulates collagens and other matrix molecules via Bax- and Bid-independent mechanisms while stimulating Egr-1, cathepsins, TIMP-1, and MMP-12, and inhibiting p21 and MMP-9 via Bax- and Bid-dependent pathways. When viewed in combination, these observations demonstrate that Bax-mediated events play critical roles in the pathogenesis of the fibrotic and inflammatory effects of TGF-\(\beta_1\). They also demonstrate that Bid is an important activator of this pathway and that the effects of this pathway are mediated, in part, by MMP-12. Because the fibrogenic effects of bleomycin were also ameliorated in mice with null mutations of Bid, Bax, or MMP-12, these studies also demonstrate that the roles of these moieties in the pathogenesis of fibrogenic tissue responses are not specific for transgenic TGF-\(\beta_1\), thereby highlighting the importance of these findings for other fibrogenic stimuli.

The TGF-\(\beta\) family proteins are multifunctional cytokines that play pivotal roles in diverse biologic processes including cell growth and survival, differentiation, development, inflammation, immunity, hematopoiesis, and tissue remodeling and repair. The complexity of these responses is such that, in some cases, they appear confusing and contradictory (14, 39–45). This can be seen in repair and remodeling responses, where it has been demonstrated that, in the proper setting, TGF-\(\beta_1\) is essential for wound healing, the stimulation of matrix molecule deposition and angiogenesis and is a critical mediator of pathologic fibrosis (14, 20, 22, 23, 37, 43, 46). On the other hand, apoptosis pathway activation plays a critical role in the pathogenesis of the TGF-\(\beta_1\)-induced apoptotic and fibrotic responses. They also provide novel mechanistic insights by demonstrating that, in the absence of Bax, the ability of TGF-\(\beta_1\) to induce epithelial apoptosis (22, 48), and an increase in the anti-apoptotic effects of p21 (49). In accord with this concept, we have already shown that p21 is an important inhibitor of TGF-\(\beta_1\)-induced epithelial apoptosis in the murine lung (3). Interestingly, these studies also demonstrate that TGF-\(\beta_1\)-induced apoptosis is only partially Bax-dependent because increased levels of apoptosis were still readily apparent in lungs from TGF-\(\beta_1\) mice with null Bax loci. This Bax-independent pathway could be mediated via a variety of apoptotic mechanisms, including the extrinsic apoptosis pathway, a Bax-independent intrinsic apoptosis pathway, an unfolded protein or endoplasmic reticulum stress response, a Bid-mediated Bax-independent response (33) or a Bak-mediated response. Additional investigations will be required to differentiate among these and other possibilities.

Alveolar remodeling and destruction are a well-described pathologic end point in a variety of pulmonary disorders including emphysema and the honeycombing of pulmonary fibrosis (50, 51). Interestingly, TGF-\(\beta_1\) and epithelial apoptosis have both been implicated in the pathogenesis of these disor-
ders (35, 52, 53). Previous studies from our laboratory demonstrated that apoptosis is a critical prerequisite for TGF-β₁-induced fibrosis and alveolar remodeling (22). The present studies add to our knowledge of these responses by demonstrating that Bax-mediated intrinsic apoptosis pathway activation plays an important role in this fibrotic, but not in the remodeling, response. This is the first demonstration that TGF-β₁ selectively uses different apoptosis pathways to engender different remodeling responses. It is also the first to dissociate TGF-β₁-induced fibrosis and tissue destruction. This has important clinical implications because it suggests that therapies can be developed that will allow physicians to control the alveolar destructive and fibrotic responses independently of one another.

In many biologic settings, whether or not a cell lives or dies is controlled by the interaction of multi-domain Bcl-2 proteins with pro-apoptotic BH3 domain-only members of this family. These BH3 domain-only molecules act as upstream sentinels that respond to proximal death and survival signals (29, 33). When activated, they neutralize or ligate anti-apoptotic Bcl-2 family proteins, thereby allowing pro-apoptotic molecules like Bax to interact with the mitochondria and induce apoptosis (29, 33). In some cases, they can also directly bind and activate Bax and other members of this family (29, 32, 33). Bid, a BH3 domain-only Bcl-2 protein, was originally discovered based on its ability to bind Bcl-2 and Bax (33). It is activated to tBid by a variety of proteases and relays its signal to Bax, which induces multiple mitochondrial alterations, including the release of cytochrome c and other apoptogenic factors (33). Because Bax is known to be activated via Bid-dependent and -independent mechanisms (38), studies were undertaken to determine if Bid played a key role in the Bax-dependent responses that were noted in our modeling system. These studies demonstrate that null mutations of Bid caused alterations in TGF-β₁-induced inflammation and remodeling that were remarkably similar to those induced by Bax ablation. This suggests that TGF-β₁ induced Bax activation is mediated, in great extent, by the ability of TGF-β₁ to activate upstream Bid molecules.

It has long been accepted that MMPs play an important role(s) in the pathogenesis of pulmonary fibrosis. However, the mechanisms of these contributions are not well understood (54, 55). Because the MMPs, as a group, have the ability to degrade a wide array of matrix molecules, it has been assumed that MMPs contribute to matrix degradation and thus inhibit...
matrix molecule accumulation. Support for this anti-fibrotic conceptualization can be seen in experimental modeling systems in which collagen deposition has been shown to correlate with decreases in MMP-9/TIMP-1 molar ratios and increases in TIMP-1 (56, 57). However, it is also becoming increasingly clear that pulmonary inflammation and remodeling are the result of several interrelated processes including extracellular matrix remodeling, basement membrane disruption, epithelial apoptosis, cell migration, and angiogenesis (54). MMPs are known to play an essential role in each of these responses, either by direct matrix molecule cleavage or by generating bioactive mediators and other biologic regulators. In accord with this complexity, circulating fibrocytes that have recently been implicated in the pathogenesis of pulmonary fibrosis are known to make MMPs (58) and interventions that block MMP-12 (and possibly other MMPs) also diminish asbestos-induced pulmonary fibrotic responses (59). As would be expected from studies cited above, our studies demonstrate that TGF-β1 inhibits MMP-9 while stimulating TIMP-1 during the generation of pulmonary fibrosis. They also demonstrate that, in the absence of Bax, the ability of TGF-β1 to inhibit MMP-9, stimulate TIMP-1 and induce tissue fibrosis is significantly diminished. Importantly, our studies highlight a previously unappreciated relationship between MMP-12, TGF-β1, and pulmonary fibrosis with TGF-β1 stimulating MMP-12 production and activation via a Bax- and Bid-dependent pathway(s) and MMP-12 playing a critical role in the pathogenesis of TGF-β1-in-duced inflammation and fibrosis in the lung. They also demonstrate that TGF-β1 stimulates MMP-12, TIMP-1 and cathepsins and inhibits MMP-9 and p21 via Bid-dependent and Bax-dependent mechanisms and that MMP-12 is an important contributor to the pathogenesis of TGF-β1-induced pulmonary fibrosis. Finally, they demonstrate that Bax, Bid, and MMP-12 mediate similar responses in bleomycin-induced fibrosis, thereby highlighting the potential importance of this regulatory pathway in other disease and injury settings. TGF-β1-induced fibrosis and apoptosis contributes to the pathogenesis of a wide variety of pulmonary and extrapulmonary diseases and disorders. These findings suggest that interventions that regulate the expression and/or function of Bax, Bid, MMP-12, or other components of this pathway, may be therapeutically useful in disorders characterized by TGF-β1-induced inflammation, fibrosis, and apoptosis. Additional investigation of the roles of this pathway in the pathogenesis of fibrotic and inflammatory disorders and the utility of Bax-, Bid-, and MMP-12-based therapeutics in their treatment is warranted.

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