Targeting Inhibitor of Apoptosis Proteins Protects from Bleomycin-Induced Lung Fibrosis

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Abstract

Accumulation of apoptosis-resistant fibroblasts is a hallmark of pulmonary fibrosis. We hypothesized that disruption of inhibitor of apoptosis protein (IAP) family proteins would limit lung fibrosis. We first show that transforming growth factor- β 1 and bleomycin increase X-linked IAP (XIAP) and cellular IAP (cIAP)-1 and -2 in murine lungs and mesenchymal cells. Functional blockade of XIAP and the cIAPs with AT-406, an orally bioavailable second mitochondria-derived activator of caspases (Smac) mimetic, abrogated bleomycin-induced lung fibrosis when given both prophylactically and therapeutically. To determine whether the reduction in fibrosis was predominantly due to AT-406-mediated inhibition of XIAP, we compared the fibrotic response of XIAP-deficient mice (XIAP^{-/-}) with littermate controls and found no difference. We found no alterations in total inflammatory cells of either wild-type mice treated with AT-406 or XIAP^{-/-} mice. AT-406 treatment limited CCL12 and IFN- γ production, whereas XIAP^{-/-} mice exhibited increased IL-1 β expression. Surprisingly, XIAP^{-/-} mesenchymal cells had increased resistance to Fas-mediated apoptosis. Functional blockade of cIAPs with AT-406 restored sensitivity to Fas-mediated apoptosis in XIAP^{-/-} mesenchymal cells *in vitro* and increased apoptosis of mesenchymal cells *in vivo*, indicating that the increased apoptosis resistance in XIAP^{-/-} mesenchymal cells was the result of increased cIAP expression. Collectively,

these results indicate that: (1) IAPs have a role in the pathogenesis of lung fibrosis; (2) a congenital deficiency of XIAP may be overcome by compensatory mechanisms of other IAPs; and (3) broad functional inhibition of IAPs may be an effective strategy for the treatment of lung fibrosis by promoting mesenchymal cell apoptosis.

Keywords: fibroblast; fibrocyte; second mitochondria-derived activator of caspases/direct inhibitor of apoptosis protein-binding protein with low pI; mesenchymal; X-linked inhibitor of apoptosis protein

Clinical Relevance

Myofibroblast resistance to apoptosis is critical for the aberrant accumulation of these cells in fibrotic lung disease. The current study demonstrates that administration of an orally bioavailable compound that functionally blocks the antiapoptotic actions of the inhibitor of apoptosis protein (IAP) family proteins, X-linked IAP, cellular IAP (cIAP)-1, and cIAP-2, enhances myofibroblast apoptosis and attenuates lung fibrosis *in vivo*. These studies support strategies targeting IAP family proteins and myofibroblast resistance to apoptosis as a therapeutic approach to fibrotic lung disease.

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Pulmonary fibrosis is characterized by the accumulation of extracellular matrix resulting in decreased lung compliance and impairment of gas exchange (1). Fibrosis can be triggered by various known insults (e.g., infection, allergens, toxins, or radiation), or can occur for unknown reasons as in the case of idiopathic pulmonary fibrosis (IPF) (2). IPF is a progressive disease with a high mortality rate and a prevalence that is increasing globally (3, 4). The pathogenesis of IPF remains unclear, but a commonly held paradigm attributes this pathology to continuous cycles of injury to the alveolar epithelium coupled with a dysregulated repair response. Prominent features of this dysregulated repair include fibroblast differentiation, myofibroblast accumulation, and excessive collagen deposition in the alveolar space (5–7). Regardless of the initial insult, the persistence of myofibroblasts is aided by their acquisition of an apoptosis-resistant phenotype (8–13).

Cell susceptibility to apoptotic stimuli is regulated, in part, by the presence of proteins that can block the propagation and execution of proapoptotic signals (14). One example is the inhibitor of apoptosis protein (IAP) family, which is composed of eight proteins: X-linked IAP (XIAP), cellular IAP (cIAP)-1, cIAP-2, melanoma IAP/Livin, IAP-like protein-2, neuronal apoptosis-inhibitory protein, Bruce/Apollon, and survivin (15). Each IAP family member contains at least one baculovirus IAP repeat domain, which allows these proteins to bind caspases and, in some cases, prevent apoptosis by directly blocking caspase activation (15–17). The most studied IAP, XIAP, is well known to inhibit the intrinsic and extrinsic apoptotic pathways through both direct mechanisms (blocking activation of caspases 3, 7, and 9) and indirect mechanisms involving its Really Interesting New Gene (RING) domain (18–24). cIAP-1 and -2 contain a similar domain structure to XIAP. Although these cIAPs do not directly inhibit caspase activation, they can impair apoptosis through alternative mechanisms (e.g., by promoting NF- κ B-induced activation of antiapoptotic proteins), and they have been shown to function cooperatively with XIAP (19, 25–27). The antiapoptotic function of XIAP and the two cIAPs can be blocked by second mitochondria-derived activator of caspases (Smac)/direct IAP-binding

protein with low pI (DIABLO), an IAP-binding protein released from mitochondria during apoptosis (28).

Recent reports indicate that XIAP is important in the pathogenesis of IPF. For example, XIAP is highly expressed within fibroblastic foci in IPF lungs (8). Also, fibroblasts from IPF lung tissue are apoptosis-resistant and show increased XIAP expression (11). Both transforming growth factor (TGF)- β 1 and another profibrotic mediator, endothelin-1, induce XIAP protein expression in normal human fibroblasts, whereas knockdown of XIAP sensitizes lung fibroblasts to Fas-mediated apoptosis (11). XIAP also plays a role in TGF- β -mediated signaling that is distinct from its direct antiapoptotic functions (29). Consistent with a profibrotic role for XIAP, treatment of lung mesenchymal cells with the antifibrotic lipid mediator, prostaglandin E₂, suppresses XIAP expression and enhances Fas-mediated apoptosis (8). Although much less is known about the role of cIAPs in lung fibrosis, based on their structural overlap with XIAP we speculate that these proteins may also influence fibroblast apoptosis resistance. We hypothesized that XIAP, and potentially the cIAPs, are critical to the pathogenesis of lung fibrosis. To address this hypothesis, we employed a murine model of lung fibrosis and antagonized the IAPs with AT-406 (an orally active mimetic of Smac/DIABLO with activity against XIAP, cIAP1, and cIAP2) and specifically disrupted XIAP via gene deletion (30).

Materials and Methods

Animals

Male wild-type C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). XIAP-deficient (XIAP^{-/-}) and wild-type littermate control mice were bred at the University of Michigan (Ann Arbor, Michigan). Because XIAP is an X-linked gene, male mice carrying a nonfunctional XIAP allele were designated as XIAP^{-/-} and are devoid of XIAP expression (31). Mice were housed under pathogen-free conditions and provided food and water *ad libitum*. All animal experiments complied with university and federal guidelines for humane use and care. The University of Michigan Committee on Use and Care of Animals approved these experiments.

Total Lung Leukocyte Preparation

Perfused whole-lung samples (all lobes) were harvested from mice, minced in PBS with protease inhibitor, and collagenase-digested as previously described (32). Centrifugation through a 40% Percoll gradient resulted in approximately 80% leukocytes, as determined by CD45 expression. Viable cells were counted on a hemocytometer by trypan blue exclusion. Differential analysis was performed as previously described (32).

Bleomycin Model of Pulmonary Fibrosis

Mice were given bleomycin intratracheally as described previously (33). In some experiments, AT-406, an orally bioactive Smac/DIABLO mimetic, which was provided by Dr. Wang's laboratory (30), was administered at a dose of 100 mg/kg by daily oral gavage starting either on Day 0 or Day 10 after bleomycin administration.

Lung Histology

Hematoxylin and eosin and Picrosirius red staining were done as previously reported (33, 34).

Lung Collagen Measurements

Collagen deposition was measured using a hydroxyproline assay on Day 21 after bleomycin challenge, as described previously (33, 34).

Semiquantitative Real-Time RT-PCR

Semiquantitative real-time RT-PCR was done as previously described (35).

ELISA

Whole-lung homogenates were prepared for analysis of cytokines and chemokines as described previously (33) using a Duoset ELISA kit (R&D systems, Minneapolis, MN).

Mesenchymal Cell Isolation

Lung mesenchymal cells were grown from lung minces for 2 weeks, at which time all cells expressed collagen 1 when analyzed by flow cytometry or immunohistochemistry (34). In some experiments, mesenchymal cells were magnetically sorted for expression of CD45 to obtain CD45⁺ collagen 1⁺ fibrocytes or CD45⁻ collagen 1⁺ fibroblasts, as previously described (36).

In Vivo Apoptosis Assessments

To assess total apoptosis in the whole-lung lysate, active caspase 3/7 levels were measured using Promega Caspase-Glo 3/7 Assay (Madison, WI), according to the manufacturer's protocol. Samples were analyzed using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) and results normalized to the PBS-treated lungs. Immunostaining for terminal deoxynucleotidyl transferase dUTP nick end labeling and α -smooth muscle actin (α -SMA) was done as previously described (34).

In Vitro Apoptosis Assay

Mesenchymal cell apoptosis was induced by treatment with activating anti-Fas antibody CH11 (Millipore, Billerica, MA) and assessed through identification of caspase 3/7 activity, as previously described (34).

Statistical Analysis

Statistical significance was assessed by ANOVA (three or more comparisons) with a Bonferroni *post hoc* test or Student's *t* test (two comparisons) using GraphPad Prism

6 software (GraphPad Software Inc., San Diego, CA). Data shown represents means ($\pm$ SEM), and a *P* value less than 0.05 was considered significant.

Results

Murine Mesenchymal Cells Have a Significant Increase in XIAP, cIAP-1, and cIAP-2 mRNA after Treatment with TGF- β 1

TGF- β 1, the central profibrotic mediator in pulmonary fibrosis, induces an apoptosis-resistant phenotype in mesenchymal cells and significantly increases XIAP expression in normal human lung fibroblasts (11). To assess whether TGF- β 1 also up-regulates IAP expression in murine mesenchymal cells, fibroblasts and fibrocytes were isolated from naive mouse lungs and treated with TGF- β 1 (2 ng/ml) in serum-free media. RNA was then isolated for real-time RT-PCR analysis. We observed that both fibroblasts and fibrocytes had significantly increased levels of XIAP, cIAP-1, and cIAP-2 mRNA in response to TGF- β 1 stimulation (Figure 1).

Functional Inhibition of IAPs with AT-406 Protects Wild-Type Mice from Bleomycin-Induced Lung Fibrosis

To examine the role of IAPs on lung fibrosis *in vivo*, we administered the orally bioavailable Smac mimetic, AT-406 (100 mg/kg by oral gavage), or vehicle control from Days 0–6 (for assessment of inflammation) or Days 0–20 (for assessment of fibrosis) to wild-type mice treated with intratracheal PBS or bleomycin on Day 0. AT-406 significantly decreased remodeling of lung architecture and fibrosis, as determined on Day 21 by hydroxyproline quantification (Figure 2A) and histology, including analysis of Picrosirius red-stained sections to elucidate collagen deposition in the lungs (Figure 2B). To determine whether AT-406 treatment impacted the recruitment of inflammatory cells, we determined the number and composition of the inflammatory cell infiltrate at Day 7 after bleomycin administration. Figure 2C demonstrates that the total inflammatory cell number was not different with AT-406

Figure 1. Transforming growth factor (TGF)- β treatment increased the expression of X-linked inhibitor of apoptosis proteins (XIAPs) and cellular inhibitor of apoptosis proteins (cIAPs) in fibroblasts and fibrocytes. Fibroblasts and fibrocytes from wild-type mice were treated with TGF- β (2 ng/ml) for 48 hours. Total RNA was isolated, and we measured the expression of XIAP (A and D), cIAP-1 (B and E) and -2 (C and F), and β -actin in fibroblasts (A–C) and fibrocytes (D–F) by real-time RT-PCR. Data represent cells collected from $n = 3$ mice total from two different experiments. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$.

Figure 2. Blockade of IAPs with AT-406 inhibits lung collagen accumulation on Day 21 after bleomycin (Bleo) treatment and decreases lung levels of chemokine (C-C motif) ligand-12 (CCL12). (A) Wild-type (WT) mice were given 1.15 U/kg of Bleo or PBS intratracheally on Day 0. AT-406 (100 mg/kg by oral gavage) was administered daily through Day 20 to half of the mice, and vehicle control (PBS) was administered to the other half. Lungs were harvested on Day 21 for hydroxyproline quantification. (B) Histochemical staining showing representative lungs of mice treated with vehicle control (PBS), Bleomycin (Bleo) or Bleo with AT-406. Shown are hematoxylin and eosin (H&E) and Picosirius red (PS). Magnification was via 4×, 10×, and 20× objectives from left to right. (C) On Day 7, lungs were harvested, digested, and total lung leukocytes were enumerated. (D) Differential analysis of leukocytes was done to determine the percentage of monocytes (Mono)/macrophages (Macs), lymphocytes (Lymphs), neutrophils (PMNs), and eosinophils (Eos). Data shown represents mean ± SEM; $n = 3$ animals/group; ns, not significant. (E) Leukocytes were plated at 3×10^6 cells/ml in serum-free media overnight. Cell-free supernatants were analyzed by ELISA for IL-1β, (F) CCL12, (G) TNF-α, and (H) IFN-γ. Data shown are pooled from two independent experiments, with $n = 4-6$ mice per group in each experiment. **** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$.

treatment. The differential analysis showed modest reductions in the percentage of monocyte/macrophages and small increases in lymphocytes and neutrophils (Figure 2D). Taken together, these data demonstrate that inhibition of XIAP together with cIAP1 and cIAP2 limited the development of fibrosis after bleomycin-induced injury without major effects on inflammation.

AT-406 Treatment Diminishes CCL12 and IFN-γ

To determine the impact of AT-406 treatment on lung cytokine levels, lung homogenates collected on Day 7 were analyzed for the expression of IL-1β, TNF-α, CCL12, and IFN-γ. Figures 2E–2H demonstrate that AT-406-treated mice showed significant inhibition of both CCL12 and IFN-γ, but no alteration in

IL-1β. There was a trend toward reduction of TNF-α.

Delayed Administration of AT-406 Has Therapeutic Benefit

To determine whether inhibition of IAP proteins could have an antifibrotic effect during the postinflammatory phase of bleomycin-induced fibrosis, we next administered AT-406 using a treatment

protocol. Wild-type mice were given bleomycin or PBS on Day 0, daily treatments with AT-406 (or vehicle) were given from Days 10–20, and lungs were harvested for analysis on Day 21. Supporting an antifibrotic effect that is independent of the early inflammatory phase, the therapeutic dosing regimen of AT-406 maintained efficacy with reductions in lung collagen to similar levels as those observed with the preventive strategy, as indicated by hydroxyproline quantification (Figure 3A), histology, and Picrosirius red staining (Figure 3B).

Therapeutic Administration of AT-406 Enhances Mesenchymal Cell Apoptosis *In Vivo*

To determine whether therapeutic AT-406 treatment enhanced apoptosis *in vivo*, AT-406 was administered beginning on Day 11 after bleomycin administration, and apoptosis was assessed on Day 13. First, the

total level of caspase 3/7 activity was measured in lung homogenates. AT-406 treatment showed a trend toward increasing caspase activity in the lung compared with the activity measured in lungs from mice treated with bleomycin and vehicle control (Figure 4A). To assess apoptosis within the myofibroblast population (α -SMA-expressing mesenchymal cells), lung sections were costained with α -SMA and terminal deoxynucleotidyl transferase dUTP nick end labeling (Figures 4B–4H). Enumeration of apoptotic myofibroblasts in stained sections revealed significantly increased myofibroblast apoptosis in the bleomycin-injured AT-406-treated mice (Figure 4B).

Genetic Deficiency of XIAP Does Not Protect against Bleomycin-Induced Lung Fibrosis

To specifically assess the role of XIAP in lung fibrogenesis, wild-type ($XIAP^{+/+}$) and

$XIAP^{-/-}$ mice were given intratracheal PBS or bleomycin on Day 0. On Day 7, lungs were collected and XIAP expression was assessed from cultured lung mesenchymal cells. These results demonstrate the up-regulation of XIAP in mesenchymal cells from the lungs of wild-type mice after intratracheal bleomycin administration (Figure 5A). As expected, mesenchymal cells from the lungs of $XIAP^{-/-}$ mice had undetectable levels of XIAP (data not shown). The remaining lungs were harvested on Day 21 and lung collagen content was measured by hydroxyproline assay in $XIAP^{+/+}$ and $XIAP^{-/-}$ mice treated with PBS or bleomycin. As expected, bleomycin-treated wild-type mice showed a significant increase in collagen content when compared with PBS controls. However, in contrast to the results with AT-406 treatment, bleomycin-treated $XIAP^{-/-}$ mice showed similar levels of lung collagen as the bleomycin-treated wild-type mice (Figure 5B). Representative histology also showed similar levels of remodeling and cellular infiltration between the two strains (Figure 5C). XIAP deficiency had no significant effect on total inflammatory cell recruitment (Figure 5D) or inflammatory cell composition (Figure 5E) at Day 7 after bleomycin.

$XIAP^{-/-}$ Mice Express More IL-1 β after Bleomycin Treatment

In contrast to the changes observed in wild-type mice treated with AT-406, the $XIAP^{-/-}$ mice had no significant changes in TNF- α , CCL12, or IFN- γ . The $XIAP^{-/-}$ mice did show enhanced production of IL-1 β (Figures 5F–5I). However, this increase in IL-1 β did not seem to impact the levels of fibrosis when compared with wild-type mice.

$XIAP^{-/-}$ Mesenchymal Cells Are Resistant to Fas-Mediated Apoptosis

Because mesenchymal cell accumulation is a critical feature of lung fibrosis, we next sought to determine how the loss of XIAP impacted mesenchymal cell apoptosis. Lung mesenchymal cells (a mixture of both fibroblasts and fibrocytes) from wild-type and $XIAP^{-/-}$ mice were treated with a Fas-activating antibody and apoptosis was assessed over time, as indicated by caspase 3/7-mediated cleavage of a fluorogenic substrate. In contrast to our findings in normal human lung fibroblasts, in which XIAP was silenced with small interfering RNA (siRNA) (11), we observed that murine lung mesenchymal cells congenitally lacking

Figure 3. Therapeutic administration of AT-406 limits lung fibrosis. (A) WT mice were given 1.15 U/kg of Bleo or PBS intratracheally on Day 0. Half of each group received AT-406 (100 mg/kg) by oral gavage daily starting on Day 10 through Day 20, and lungs were harvested on Day 21 for hydroxyproline quantification. (B) Histochemical staining showing representative lungs of mice treated with intratracheal PBS and vehicle control (PBS), intratracheal Bleo and AT-406, intratracheal Bleo and vehicle control, or intratracheal Bleo and AT-406. Shown are H&E and PS staining. Magnification was via 20 $\times$ objective. Data shown represent mean $\pm$ SEM, $n = 4$ –6 mice/group. **** $P < 0.0001$ and * $P < 0.05$.

Figure 4. AT-406 augments myofibroblast apoptosis *in vivo* after Bleo injury. WT mice were given 1.15 U/kg of Bleo or PBS intratracheally on Day 0. On Days 11, 12, and 13, half of each group was administered AT-406 (100 mg/kg via oral gavage), and the lungs were harvested on Day 13. (A) Whole-lung homogenates were assessed for activation of caspase 3/7. $P < 0.01$ for Bleo/AT-406 compared with controls. (B–H) Lung sections were costained for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (C and F) and α -smooth muscle actin (SMA; D and G). Cells that were positive for both TUNEL and α -SMA were quantified using merged images (E and H) in four random 20 $\times$ objective fields by an investigator who was blinded to the treatment groups. This quantification (B) demonstrated significantly more copositive cells in Bleo/AT-406 lung sections compared with Bleo/PBS lung sections. $P = 0.01$. Costained cells in representative merged images (E and H) are indicated by arrows. Data shown represent mean $\pm$ SEM.

XIAP had increased resistance to Fas-mediated apoptosis when compared with wild-type cells (Figure 6A). This finding suggested that alternative antiapoptotic mechanisms might provide functional compensation for the genetic deficiency in XIAP^{-/-} mice, and offered a potential explanation for why these mice remain susceptible to bleomycin-induced fibrosis.

cIAPs Expression Is Increased in XIAP Gene-Targeted Mice

Initial characterization of the XIAP^{-/-} mice demonstrated that the expression of cIAP-1 and -2 proteins was increased, suggesting that, in the congenital absence of XIAP, there exists a mechanism by which the increased production of other IAP family members may contribute to functional

compensation (31). We analyzed the expression of cIAP-1 and -2 in lung homogenates and in lung mesenchymal cells cultured from XIAP^{+/+} and XIAP^{-/-} mice treated with bleomycin. In the lung homogenates, levels of cIAP-1 were significantly elevated after intratracheal bleomycin in wild-type and XIAP-deficient mice, and cIAP-2 showed a nonstatistically significant increase in XIAP^{-/-} mice (Figures 6B and 6C). In isolated mesenchymal cells from untreated mice, however, the opposite result was seen, with basal levels of cIAP-1 being unchanged (Figure 6D), whereas cIAP-2 mRNA expression was significantly increased in XIAP^{-/-} cells when compared with wild-type (Figure 6E). These data support the possibility of a functional compensation at baseline by cIAPs in the XIAP^{-/-} mice.

AT-406 Sensitizes XIAP^{-/-} Mesenchymal Cells to Fas-Mediated Apoptosis

To determine if increased cIAP expression might provide functional compensation for the deficiency of XIAP in lung mesenchymal cells, we determined whether inhibition of cIAP-1 and cIAP-2 would restore sensitivity to Fas-induced apoptosis in XIAP^{-/-} mesenchymal cells. XIAP^{-/-} cells were treated with the Smac mimetic, AT-406, and susceptibility to Fas-mediated apoptosis was assessed over 24 hours. Supporting the hypothesis of functional compensation by cIAPs, inhibition of cIAP-1 and -2 with AT-406 enhanced the apoptotic sensitivity of the XIAP-deficient mesenchymal cells (Figure 6F). Of note, treatment of XIAP-deficient mesenchymal cells with AT-406 alone did not increase apoptosis in the absence of Fas-activation, suggesting that the increased apoptosis was not a nonspecific effect of the compound.

AT-406 Treatment Limits Fibrosis in XIAP^{-/-} Mice

To verify that AT-406 treatment could reduce bleomycin-induced fibrosis *in vivo*, XIAP^{-/-} mice or XIAP^{+/+} mice were treated with intratracheal PBS or bleomycin and AT-406 or vehicle control beginning on Day 0. There was an unexpected early toxicity of AT-406 treatment that was observed during the acute inflammatory phase of the model (between Days 4 and 7) in the cohort of XIAP^{-/-} mice that had received intratracheal bleomycin. This effect was observed in multiple experiments

Figure 5. XIAP-deficient mice are not protected from Bleo-induced pulmonary fibrosis and show elevated levels of IL-1 β . WT littermate (XIAP^{+/+}) or XIAP-deficient (XIAP^{-/-}) mice were given Bleo or PBS intratracheally on Day 0. Lungs were harvested on Day 7. Lung minces were then digested in collagenase, total lung leukocytes were enumerated, and lung mesenchymal cells were cultured. (A) XIAP mRNA expression was assessed in cultured lung mesenchymal cells by RT-PCR. (B) On Day 21, lungs were harvested for hydroxyproline quantification. (C). Representative H&E staining from Day 21. Magnification was through the 10 $\times$ (top) and 20 $\times$ (bottom) objectives. Representative of $n = 4$ mice examined. (D) Total lung leukocytes from Day-7 lung minces were enumerated. (E) Differential analysis was done to determine the percentage of Mono/Macs, Lymphs, PMNs, and Eos. Data shown represent $n = 6$ –10 mice per group pooled from three independent experiments. Cells were plated at 3×10^6 cells/ml in serum-free media overnight, and cell-free supernatants were analyzed by ELISA for IL-1 β (F), TNF- α (G), CCL12 (H), and IFN- γ (I). Data shown represent mean $\pm$ SEM; $n = 4$ –6 mice/group. **** $P < 0.0001$, ** $P < 0.01$.

and reduced the n value for these experiments. However, in the mice that did survive the inflammatory phase of the model, there was a trend toward reduced levels of collagen accumulation in the lungs, as measured by hydroxyproline assay (Figure 7A). Analysis of accumulated inflammatory cells and cytokines at Day 7 showed no changes other than increased neutrophils in the XIAP^{-/-} mice that

received bleomycin, which was accentuated in the XIAP^{-/-} mice that received the bleomycin and AT-406, suggesting that toxicity in this group might be related to alterations in the acute response to injury (Figures 7B–7E). In addition, AT-406 treatment was associated with a trend for decreased elaboration of IL-1 β and CCL12 from leukocytes isolated from the XIAP-deficient mice on Day 7 after bleomycin

(Figures 7F and 7G). AT-406 treatment did not alter TNF- α or IFN- γ secretion by these leukocytes (data not shown).

Discussion

Previous studies have shown that fibroblastic foci, but not the epithelial cells in IPF tissues, express high levels of XIAP

Figure 6. XIAP^{-/-} mesenchymal cells have increased cIAP expression associated with decreased susceptibility to Fas-mediated apoptosis, and inhibition of cIAPs enhances their apoptosis. (A) Lung fibroblasts from WT and XIAP^{-/-} mice were treated with/without Fas-activating antibody (250 ng/ml) along with Cellplayer kinetic caspase 3/7 reagent. Plates were loaded into the IncuCyte incubator (Essen Bioscience, Ann Arbor, MI) and photographed every 2 hours for 24 hours. Apoptosis was quantified by automated counting “objects per well” (object = green fluorescence indicating cleavage of a fluorogenic substrate by caspase 3/7). (B and C) Total RNA was isolated from fibroblasts cultured from the lungs of XIAP^{+/+} and XIAP^{-/-} mice treated with/without Bleo and expression of cIAP-1 (A), cIAP-2 (B), and β -actin was measured by real-time RT-PCR. Data represent $n = 3$ per group pooled from multiple mice. ** $P < 0.01$. (D and E) RNA isolated from lung homogenates of untreated XIAP^{+/+} and XIAP^{-/-} mice were assessed for cIAP-1 and -2. Data represent $n = 3$ per group pooled from multiple mice. * $P < 0.05$. (F) Lung fibroblasts from XIAP^{-/-} were treated with/without Fas-activating antibody (250 ng/ml) and/or AT-406 (1.0 μ M). Apoptosis was evaluated as described above with $n = 3$ wells/group. * $P < 0.05$ versus control and AT-406 and ** $P < 0.01$ versus Fas treatment. Data represent mean $\pm$ SEM; $n = 3$ wells/treatment per group, with nine images per well at each time point.

(8, 37), that prostaglandin E₂ suppresses XIAP expression while increasing fibroblast susceptibility to apoptosis, that the profibrotic mediators, TGF- β 1 and endothelin-1, increase XIAP expression in normal fibroblasts, and that XIAP (but not cIAP-1 or

cIAP-2) expression is increased in lung fibroblasts from patients with IPF (8, 11). Together, these studies suggested that XIAP might contribute to the accumulation of myofibroblasts and extracellular matrix in IPF, and that targeting XIAP could represent

a therapeutic approach for lung fibrosis. Testing this hypothesis in a murine model of experimental lung fibrosis using pharmacologic and genetic approaches led to discordant findings *in vivo*, and our subsequent studies suggested that other IAP

Figure 7. AT-406 decreases Bleo-induced lung fibrosis in XIAP^{-/-} mice. (A–I) XIAP^{+/+} or XIAP^{-/-} mice were given Bleo or PBS intratracheally on Day 0. Lungs were harvested and assessed for inflammation on Day 7 and fibrosis on Day 21, as described in Figure 5. (A) Hydroxyproline quantification at Day 21. Data shown represent mean $\pm$ SEM; $n = 7$ –11 mice/group. **** $P < 0.0001$, * $P < 0.05$. (B–E) Leukocyte differentials in lung collagenase digest on Day 7 for macrophages (B), lymphocytes (C), neutrophils (D), and eosinophils (E); $n = 3$ lungs per group. * $P < 0.05$. (F–G) Cytokines in cell-free supernatants from Day-7 leukocytes for IL-1 β (F) and CCL12 (G).

family members (i.e., the cIAPs) may contribute to mesenchymal cell apoptosis resistance and fibrogenesis.

Consistent with our findings in human lung fibroblasts, we found that the profibrotic mediator, TGF- β 1, enhanced expression of XIAP in murine mesenchymal cells. Importantly, TGF- β 1 also increased the expression of cIAPs (cIAP-1 and -2) in these cells. Similarly, bleomycin treatment up-regulated XIAP and cIAP-1 as well. Treatment of mice with the Smac mimetic, AT-406, substantially decreased lung fibrosis *in vivo*, establishing a causal role for the IAPs in lung fibrogenesis. In contrast to the broad inhibition of the IAPs with AT-406, the specific targeting of XIAP by transgenic deletion did not protect against bleomycin-induced fibrosis. Assessment of early inflammatory responses to bleomycin-treated mice showed no differences in total inflammatory cell recruitment in the mice

treated with AT-406 or in XIAP^{-/-} mice compared with wild-type mice treated with bleomycin. Differential cell counts showed no difference between inflammatory cell populations in wild-type and XIAP-deficient mice that were not protected from fibrosis; in contrast, mice receiving AT-406 did have a small, but statistically significant, decrease in the monocyte/macrophage population coupled with increases in the lymphocyte and neutrophil populations. Thus, it seems unlikely that the protection from fibrosis seen by AT-406 administration is due to decreased lung injury and inflammation. In fact, we were surprised to find that AT-406 treatment in the XIAP^{-/-} mice actually showed some toxicity and enhanced neutrophil recruitment.

When analyzing the cytokine profiles of XIAP^{-/-} or AT-406-treated mice, the XIAP^{-/-} mice showed increases in IL-1 β , but this did not alter levels of lung fibrosis.

In contrast, the AT-406-treated mice had equivalent levels of IL-1 β , but did show reduced expression of CCL12, IFN- γ , and a trend for decreased TNF- α . Certainly, CCL12 has been shown to promote fibrotic responses in the lung by mediating fibrocyte recruitment (36), so reduced levels of CCL12 may limit fibrocyte accumulation. Similarly, the CCL12 receptor, CCR2, has been shown to be critical for the recruitment of exudate macrophages to the lung, and decreased recruitment of exudate macrophages was associated with decreased fibrosis in a model of targeted type II alveolar epithelial injury (38). The impact of IFN- γ on lung fibrogenesis is controversial, and clinical trials have failed to demonstrate a significant therapeutic benefit for IFN- γ as a treatment for IPF (39, 40). However, as these clinical trials were predicated on the multitude of antifibrotic actions of IFN- γ in preclinical models, it seems unlikely that the decline in

IFN- γ observed with AT-406 treatment accounts for the decreased fibrosis observed in our experiments. Thus, while acknowledging that differences in the accumulation of monocyte/macrophages and fibrocytes, or decreased levels of CCL12 or IFN- γ , might contribute to the discordant fibrotic outcomes in these two approaches, we went on to investigate the apoptotic susceptibility of murine lung mesenchymal cells.

Studies increasingly support the hypothesis that impaired apoptosis contributes to myofibroblast accumulation in lung fibrosis (8, 11–13, 41–43). Consistent with the initial characterization of XIAP-deficient mice (31), our investigation revealed that mesenchymal cells from the XIAP-deficient mice did not demonstrate increased susceptibility to Fas-mediated apoptosis. Indeed, the cells lacking XIAP actually had a significant decrease in apoptotic susceptibility. Because XIAP^{-/-} mesenchymal cells maintained expression of cIAP-1 and displayed enhanced expression of cIAP-2, we interpreted these results to suggest that alternative antiapoptotic mechanisms, including the increase in cIAP protein expression, might provide a functional compensation for the genetic loss of XIAP. Consistent with this hypothesis, treatment of XIAP^{-/-} mesenchymal cells with AT-406 restored the anticipated apoptotic

susceptibility to XIAP^{-/-} cells *in vitro*, and treatment of the bleomycin-treated mice with AT-406 increased myofibroblast apoptosis *in vivo*. Because resistance to apoptosis contributes to the persistence of myofibroblasts, we speculate that the different outcomes to bleomycin-induced lung injury are related to the differential apoptotic susceptibility of mesenchymal cells in these different genetic and pharmacologic models. If true, it is encouraging that AT-406 was able to limit fibrosis after bleomycin challenge. Because Smac mimetic compounds are already being tested in clinical trials as cancer therapeutics (18, 44–48), such therapies could potentially be adopted quickly in IPF.

The fact that AT-406 exerted beneficial effects that were not mirrored by XIAP deficiency, and the fact that cIAP proteins do not antagonize caspase activation (20, 49, 50), raises the question of how the cIAP proteins may be functioning to promote bleomycin-induced fibrosis. Certainly, this family of proteins functions through a variety of mechanisms involving TGF- β signaling, NF- κ B activation, and E3 ligase activity (27, 29, 51). cIAP1 and -2 have been shown to be important in TNF- α -mediated NF- κ B activation (52); however, we did not observe a significant difference in TNF- α *in vivo*. Thus, it is likely that cIAP-1 and -2 contribute to the antiapoptotic phenotype via induction of other antiapoptotic proteins,

or via modulation of cell signaling pathways yet to be determined. Importantly, AT-406 acts to promote degradation of cIAP-1 and -2, raising additional mechanistic possibilities for the antifibrotic actions of this approach (53–55).

XIAP is expressed in the fibroblastic foci of IPF tissues and is increased in IPF fibroblasts, but cIAP-1 and -2 are not increased in IPF fibroblasts (8, 11). This suggests that, in human IPF fibroblasts, there may be less compensation than is occurring in XIAP^{-/-} mice, or that cIAP protein function is sufficient at levels present in IPF cells. The potential for cIAPs to compensate for the postnatal loss or inhibition of XIAP in adults has yet to be explored. Regardless, it is encouraging that AT-406 has previously been shown to restore apoptotic sensitivity of IPF fibroblasts to Fas-mediated apoptosis (11), and that our murine studies show benefit of AT-406 when given therapeutically during the fibroproliferative phase of the disease. ■

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