Inverse Effects of Interleukin-6 on Apoptosis of Fibroblasts from Pulmonary Fibrosis and Normal Lungs


Asthma and Allergy Research Institute, Sir Charles Gairdner Hospital, Nedlands, Western Australia; School of Medicine and Pharmacology, University of Western Australia, Crawley, Western Australia, Australia; and Centre for Cardiopulmonary Biochemistry and Respiratory Medicine, Royal Free and University College Medical School, University College London, London, United Kingdom

Fibroblast apoptosis is crucial to the resolution of fibrosis. However, the mechanisms by which these cells undergo apoptosis are not well known. Because interleukin (IL)-6 and IL-11 may alter repair and remodeling processes, we hypothesized that they may play a role in idiopathic pulmonary fibrosis (IPF). We investigated the effects of these cytokines on Fas-induced apoptosis using primary lung fibroblasts from three patients with IPF (IPF-Fb) and three subjects without lung disease (normal-Fb). IPF-Fb were resistant to Fas-induced apoptosis compared with normal-Fb ($P < 0.01$). Using RNase protection assays, we showed that IL-6 enhanced Fas-induced apoptosis and expression of Bax in normal-Fb, but inhibited apoptosis and induced expression of Bcl-2 in IPF-Fb. Densitometry of Western blots revealed a Bcl-2/Bax ratio of $0.15 \pm 0.01$ in normal-Fb compared with $1.05 \pm 1.0$ in IL-6-Fb. Upregulation of Bcl-2 in normal-Fb and Bax in IPF-Fb were both STAT-3-dependent. Inhibition of extracellular signal–regulated kinase had no effect in normal-Fb, but reversed the antiapoptotic effect of IL-6 in IPF-Fb. IL-11 inhibited Fas-induced apoptosis and increased Bcl-2 expression in both normal-Fb and IPF-Fb. These results suggest that altered IL-6 signaling in IPF-Fb may enhance the resistance of these cells to apoptosis and contribute to a profibrotic effect of IL-6 in IPF.

Idiopathic pulmonary fibrosis (IPF) is a chronic, largely untreatable, diffuse lung disease of unknown etiology (1–4). Morphologically, the disease is characterized by abnormal parenchymal tissue remodeling and re-epithelialization and increased collagen deposition (5–7). The progression of fibrosis appears to be strongly correlated with the presence and number of myofibroblastic foci in the injured lung (4).

Myofibroblasts are characterized by the expression of markers of smooth muscle differentiation such as α-smooth muscle actin (α-SMA), and are thought to be the major source of collagen and profibrogenic growth factors within the lung (8, 9). Under normal conditions, myofibroblasts are transient but essential cells in the resolution of inflammation and scar formation, and are cleared from the wound site by apoptosis (10, 11). We and others have shown that primary cultures of fibroblasts from patients with IPF (IPF-Fb) contain significantly greater numbers of myofibroblasts compared with cultures from subjects without lung disease (normal-Fb). This, together with observations of an increased number of myofibroblasts in IPF lungs, suggests that these cells may be resistant to apoptosis.

The cellular mechanisms specifically involved in myofibroblast apoptosis are not well known. One group of proteins, the Bcl-2 family, has been widely studied and consists of members that suppress (Bcl-2) or activate (Bax) apoptosis. Indeed, the balance of these pro- and antiapoptotic pathways may dictate whether a cell survives or undergoes apoptosis. Altered expression of these proteins, therefore, has the potential to confer resistance to physiologic signals for apoptosis.

Under normal conditions, the progressive repair of damaged tissue and clearance of apoptotic cells is orchestrated by a cascade of cytokines and growth factors, including interleukin (IL)-1β (proapoptotic) and transforming growth factor-β (antiapoptotic) (11). However, the regulatory role and relative importance of more recently identified cytokines needs to be assessed. IL-6 and IL-11 belong to a family of pleiotrophic cytokines that affects a variety of biological functions related to inflammation (12, 13). Signaling initiated by this cytokine family occurs by association of their respective specific α-subunits with the ubiquitous signal transducer gp130. This complex tyrosine phosphorylates janus kinases (JAK), leading to recruitment of adapter molecules such as signal transducer and activator of transcription (STAT)-3 and SHP-2, which permits activation of the Ras-raf-ERK pathway. The interplay between SHP-2 and STAT-3 in mediating signals through gp130 has been implicated in growth, differentiation, and apoptosis of a number of cell types.

IL-6 is released by a variety of cells, including fibroblasts, and has been shown to mediate many inflammatory processes in the lung (13), and its dysregulated release has been implicated in the pathogenesis of a variety of respiratory conditions including interstitial lung diseases (14). However, its role in fibrosis is unclear. In animal models, lung-specific
overexpression of IL-6 produces inflammation but little fibrosis (15, 16). However, several genetic studies in both animals and humans have shown strong association between IL-6 and the development of fibrosis (17, 18). We have recently shown that IL-6 has contrasting effects on the mitogenic responses of normal-Fb and IPF-Fb. In normal cells, IL-6 induced growth arrest and inhibited proliferation, whereas in IPF-Fb, IL-6 was mitogenic. This was associated with an altered dependency on STAT-3 and ERK signaling (19). Little is known about the role of IL-6 in fibroblast apoptosis. In transformed cell lines and tumor cells, IL-6 has been shown to be both pro- and antiapoptotic by regulating the expression and activity of the bcl-2 family of proteins (20).

IL-11 is also released by numerous cell types, including fibroblasts, and its synthesis is induced by a number of factors, including transforming growth factor-β (21, 22). IL-11 attenuates the proapoptotic response of epithelial cells to hyperoxia (23) and radiation-induced injuries (24). However, little is known about the mechanisms involved and whether similar processes occur in lung fibroblasts.

Based on these findings, we hypothesized that IL-6 may promote and IL-11 may inhibit fibroblast apoptosis. We tested this hypothesis using fibroblasts from three normal (normal-Fb) and three patients with IPF (IPF-Fb).

Materials and Methods

Materials
Dulbecco’s modified Eagle’s medium (DMEM), penicillin, gentamicin, amphotericin, and recombinant human IL-11 were purchased from Invitrogen (Melbourne, Victoria, Australia). Annexin V–fluorescein isothiocyanate and TUNEL reagents were purchased from Roche Diagnostics (Sydney, NSW, Australia). FasL was purchased from Calbiochem (Sydney, NSW, Australia). Protopidium iodide and recombinant human IL-6 were purchased from Sigma Chemicals (St. Louis, Mo). Labtec coverslip chamber wells were obtained from NUNC (Roskilde, Denmark). Genestein, wortmannin, pp2, and PD98059 were purchased from Biomol (Plymouth, PA). The RNase protection assay (RPA) for the Bcl-2 (hApo-1) was purchased from Pharmingen (Sydney, NSW, Australia). Monoclonal antibodies to Bcl-2 and Bax were purchased from Boehringer Mannheim and Dako Corporation, respectively (Sydney, NSW, Australia). [α-32P]-UTP was obtained from Amersham (Sydney, NSW, Australia).

Cell Culture
Primary cultures of fibroblasts were derived from normal human lung and patients with the usual interstitial pneumonitis (UIP) form of IPF as previously described (25). Three primary lines of normal human alveolar lung fibroblasts were obtained from Clonetics (San Diego, CA), ATCC (Manassas, VA), and lung transplant donors, respectively. Normal cell lines were used between passages 2 and 16. For comparison, primary cultures of lung fibroblasts harvested from three patients with UIP-IPF were used for all experiments. All IPF-Fb were used between passages 3 and 6. Fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum, penicillin, gentamicin, and amphotericin. For all experiments, fibroblasts were quiesced by the addition of serum-free DMEM for 16 h, before treatment with IL-6 or IL-11.

Antisense Oligonucleotides
Phosphothiorated 21-mer oligonucleotides were synthesized on an Applied Biosystem 394 synthesizer by means of β-cyanoethylphosphoramide chemistry to minimize degradation by endogenous
nucleases. The antisense oligonucleotides (ASON) were directed against the translation start site (AUG codon) and surrounding nucleotides of the human STAT-3 and STAT-5 genes. The sequence of the ASON to STAT-3 was 5'-CCATTGGGCCATCCTGTTTCT-3' and the corresponding sense oligonucleotide sequence was 5'-AGAAACAGGATAACCCAATGG-3'. The sequence of the ASON to STAT-5 was 5'-AGCCCGCCAT-3' and the corresponding sense oligonucleotide sequence was 5'-ATGGCGGGCT-3'. To examine the effect of ASON, cells were cultured at a density of 10^4 cells/well in a 96-well plate for 24 h. Cells were incubated with 250 μl of fresh serum-free medium containing 12.5 μM of either antisense or sense oligonucleotides for 24 h. Following incubation with oligonucleotides, fibroblasts were treated with IL-6 or IL-11 for 3, 6, 24, or 48 h.

**Flow Cytometry**

**Assessment of apoptosis.** Fibroblasts were quiesced in serum-free DMEM for 16 h, then treated with IL-6 or IL-11. Following exposure to IL-6 or IL-11, fibroblasts were rendered apoptotic by exposure to FasL (50 μg/ml) for 24 h. Assessment of apoptosis was performed as described previously (26). Fibroblasts were defined as apoptotic on the basis of positive staining with annexin V–fluorescein isothiocyanate. Cells positively stained with propidium iodide (PI) were excluded as being necrotic.

**Western blot analysis.** The expression of Bcl-2 and Bax was assessed by Western blot analysis. Trypsinised cells (2 × 10^6) were lysed in 50 mM Tris, 0.5 mM EGTA, 150 mM NaCl, 1% Triton X-100, pH 7.5 containing a cocktail of protease inhibitors and centrifuged at 13,000 rpm for 20 min. The protein content of the resultant supernatant was determined by the Bradford method. Equal amounts of protein (40 μg) were boiled for 5 min in sodium dodecyl sulfate–polyacrylamide gel electrophoresis buffer and electrophoresed on an 8–16% gradient gel. Following separation, proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Nonspecific binding was blocked by incubation in Tris-buffered saline–Tween 20 (TBST) containing 5% skim milk powder. Membranes were then incubated with primary antibodies against either Bcl-2 or Bax for 1 h. After washing in TBST, membranes were incubated with horseradish peroxidase–conjugated secondary antibody, washed, and proteins detected by enhanced chemiluminescence. Relative protein expression induced by cytokine treatment was assessed by densitometry using ImageQuant software (version 5.1; Molecular Dynamics, Sunnyvale, CA) and expressed in arbitrary units. Membranes were stripped and reprobed with a monoclonal antibody against tubulin as a loading control.

**RNase protection assay.** Fibroblasts were treated with either IL-6 or IL-11 after quiescing for 16 h in serum-free medium. Following trypsinization, total cellular RNA was extracted from 1 × 10^6 fibroblasts using an RNeasy mini kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. The purity of the RNA was assessed from the A260/A280 ratio and a total of 20 μg was used for the assay. Probe synthesis, hybridization, proteinase K, and RNase digestion were performed according to the manufacturer’s protocol. Samples were resolved on 5% acrylamide gels that were dried at 80°C under vacuum. The gels were then placed on autoradiographic film and incubated for 24 h before development. Band densities were analyzed using ImageQuant.
software after scanning the films on a Typhoon phosphoimager (Amersham).

Statistical Analysis

For comparison between cell lines, data were expressed as mean ± SE values for each of the three IPF-Fb or normal-Fb cultures. At least three experiments were performed in triplicate on each cell line. Statistical comparisons of mean grouped data were performed using one-way ANOVA with post hoc Bonferroni correction to correct for multiple comparisons. A P value < 0.05 was regarded as statistically significant.

RESULTS

IPF-Fb Are Resistant to Apoptosis Compared with Normal-Fb

Immunofluorescence analysis of fibroblast cultures at similar passage revealed that 67 ± 6% of the IPF-Fb expressed high levels of α-SMA compared with 2 ± 0.5% for normal-Fb.

Both IPF-Fb and normal-Fb underwent apoptosis following exposure to FasL. However, the percentage of IPF-Fb that underwent FasL-induced apoptosis over a 24-h period was much lower than that of normal-Fb (15 ± 0.5% versus 25 ± 3%; P < 0.01) (Table 1).

IL-6 Promotes Apoptosis of Normal-Fb but Inhibits Apoptosis of IPF-Fb, whereas IL-11 Is Antiapoptotic in Both Cell Types

Exposure to IL-6 significantly enhanced FasL-induced apoptosis of normal-Fb over a 48-h period compared with control cells that were exposed to FasL alone, whereas in IPF-Fb, IL-6 significantly inhibited FasL-induced apoptosis as determined by Annexin V staining (Figure 1A) or Tunel positivity (Figure 1C). In contrast to the differential effects of IL-6, exposure to IL-11 significantly inhibited Fas-induced apoptosis in both normal-Fb and IPF-Fb (Figures 1B and 1D).

The Role of STAT and Mitogen-Activated Protein Kinase Pathways on Fibroblast Apoptosis Induced by IL-6 and IL-11

The proapoptotic effects of IL-6 on normal-Fb were completely abrogated by ASON to STAT-3 (Figure 2A). However, the effects of IL-6 were not inhibited by PD98059 or nimesulide, suggesting that p42/p44 mitogen-activated protein kinase and COX-2 metabolites were not involved (Figure 2B).

In IPF-Fb, ASON to STAT-3 substantially reversed the antiapoptotic effects of IL-6 (Figure 2C). However, in contrast to normal-Fb, inhibition of ERK with PD98059 also
IL-6 Activates STAT-3–Dependent Production of Bax in Normal-Fb but Bcl-2 in IPF-Fb

We sought to identify possible pro- and antiapoptotic Bcl-2 family proteins that might be induced by IL-6 in fibroblasts. By RPA, we demonstrated mRNA expression for a number of members of the Bcl-2 family in both normal-Fb and IPF-Fb following exposure to either IL-6 or IL-11. Notably, mRNA for the proapoptotic protein Bax was markedly induced by IL-6 in normal-Fb, whereas expression of mRNA for the antiapoptotic molecule Bcl-2 was induced in IPF-Fb (Figures 4A and 4B). In contrast, IL-11 induced the expression of Bcl-2 mRNA in both normal-Fb and IPF-Fb (Figures 4A and 4B).

These observations were supported by experiments showing that Bcl-2 and Bax proteins were also differentially expressed following exposure to IL-6, with Bax upregulation in normal-Fb but Bcl-2 upregulation in IPF-Fb (Figure 5). The effects of IL-6 on both cell types were completely abrogated by ASON to STAT-3 (Figure 6).

Figure 4. The effect of IL-6 and IL-11 on expression of mRNA for members of the Bcl-2 family in normal-Fb and IPF-Fb. Fibroblasts were treated with either IL-6 or IL-11 after quiescing for 16 h in serum-free medium. A. Comparative analysis of mRNA expression for members of the Bcl-2 family was assessed using a riboquant hApo-1 RPA kit. B. Densitometric analysis of scanned blot. Exposure of normal-Fb to IL-6 resulted in the selective expression of the proapoptotic molecule Bax (filled bars). Exposure to IL-11 induced an increase in the expression of the antiapoptotic molecule Bcl-2 (open bars). In IPF-Fb, both IL-6 and IL-11 induced the expression of Bcl-2. *P < 0.01 compared with control cells.

DISCUSSION

It is increasingly being recognized that the pathogenesis of IPF is directly related to the presence and activity of myofibroblasts (6, 27). Equally, the persistence of the fibrotic lesions that characterize IPF suggests that decreased apoptosis of these cells may play a major role in this disease. In this study, we have examined the effects of IL-6 and IL-11 on FasL-induced apoptosis of normal lung fibroblasts compared with fibroblasts from patients with UIP-IPF. The study has identified several significant differences between normal-Fb and IPF-Fb, and has investigated the signal transduction events that might explain these differences. We observed that (i) IPF-Fb had a lower rate of spontaneous and FasL-induced apoptosis compared with normal-Fb; (ii) in normal-Fb, IL-6 enhanced FasL induced apoptosis, whereas in IPF-Fb, IL-6 was a potent inhibitor of apoptosis; reversed the antiapoptotic effect of IL-6 in these cells (Figure 2D). As was observed for normal-Fb, treatment of IPF-Fb with nimesulide did not influence the effect of IL-6 (Figure 2D).

The inhibitory effect of IL-11 on FasL-induced apoptosis in both normal-Fb and IPF-Fb was substantially reversed by ASON to STAT-5a/5b (Figures 3A and 3B). Furthermore, the effect of IL-11 also appeared to involve ERK, because pretreatment with PD98059 reversed the antiapoptotic effect seen with this cytokine (Figures 3C and 3D).
III) IL-6, acting via a STAT-3–dependent mechanism, preferentially induced the expression of the proapoptotic molecule Bax in normal-Fb, whereas it induced expression of the antiapoptotic protein Bcl-2 in IPF-Fb; (iv) IL-11 was antiapoptotic in both normal-Fb and IPF-Fb and this effect was mediated by a STAT-5–dependent upregulation of Bcl-2. These results provide strong evidence that a fundamental abnormality in the IL-6 signaling pathway may play an important role in the pathogenesis of IPF.

Our observation that IPF-Fb had a reduced rate of Fas-induced apoptosis compared with normal-Fb is in agreement with recent observations of Tanaka and coworkers (27), who showed that resistance to Fas-induced apoptosis in human primary lung fibroblasts is associated with the expression of antiapoptotic proteins such as X chromosome–linked inhibitor of apoptosis and FLICE-like inhibitor protein. Furthermore, the expression of these proteins was upregulated in the lungs of patients with IPF. Thus, increased expression of antiapoptotic proteins by IPF-Fb may confer some resistance to Fas-induced apoptosis, and our findings suggest that increased production of IL-6 in IPF would further reduce this inherently low rate of apoptosis.

IL-6 and IL-11 belong to a closely related family of cytokines that are produced by a number of cell types in response to inflammatory stimuli (28). These cytokines bind to specific cell surface receptors that have a cytokine specific \( \alpha \) chain and a shared gp130 signal transducing subunit (13, 29). Association of the IL-6 receptor-gp130 complex results in the simultaneous activation of both the JAK/STAT and shp2-Ras-Raf-ERK signal transduction pathways, and the integration of these pathways produces diverse, cell type–specific effects on cell growth and survival (30, 31).

Although we and others have shown that some members of this cytokine family have profibrotic properties (23, 29), the role of IL-6 in inducing fibrosis is unclear. In animal models, targeted overexpression of IL-6 in the lung does not appear to induce marked fibrosis (15, 16). However, several genetic studies using both animals and humans have shown associations between IL-6 and fibrosis. For example, in a mouse model of bleomycin-induced fibrosis, segregation between strains susceptible or resistant to fibrosis were linked to differences in the level of inducibility of IL-6 mRNA expression (17). More recently, Pantelidis and colleagues (18) showed a strong independent association between polymorphisms in the IL-6 gene and lower carbon monoxide transfer (DL\(_{CO}\)) in patients with IPF. In contrast, there was no association with polymorphisms in the genes for tumor necrosis factor-\( \alpha \), tumor necrosis factor-\( \alpha \)RII, or lymphotixin.

In this study, we have demonstrated that in normal-Fb, IL-6 enhances FasL-induced apoptosis by a STAT-3–dependent mechanism that results in increased expression of Bax mRNA and protein and a correspondingly low Bcl-2/Bax ratio. In contrast, treatment of IPF-Fb with IL-6 conferred resistance to FasL-induced apoptosis. Although this effect was also mediated by activation of STAT-3, this did not result in increased expression of Bax but rather an increased expression of Bcl-2 mRNA and protein, resulting
Figure 6. The influence of ASON on the effects of IL-6 and IL-11 in normal-Fb and IPF-Fb. Fibroblasts were treated with either IL-6 or IL-11 after quiescing, as described in Figure 4. Expression of Bel-2 family proteins was assessed by Western blot analysis. In normal-Fb, IL-6 induced the expression of Bax protein, whereas IL-11 induced expression of Bel-2 protein. The effects of IL-6 on Bax expression were completely inhibited by pretreatment with ASON to STAT-3. In IPF-Fb, both IL-6 and IL-11 induce the expression of Bel-2 protein. Pretreatment with ASON to STAT-3 blocked the effects of IL-6, whereas ASON to STAT 5 inhibited the effects of IL-11.

in a high Bel-2/Bax ratio. The induction of both Bel-2 and Bax following activation of STAT-3 has been shown in hemopoietic cells and transformed cell lines (32, 33), but this study is the first to demonstrate a differential, disease-dependent expression of these proteins.

Although the precise mechanisms underlying the difference in Bel-2/Bax ratio between IL-6–treated normal-Fb and IPF-Fb remain to be elucidated, our results suggest that differential activation of the Ras-Raf-ERK pathway and its interaction with STAT-3 may be involved. We have recently shown that in normal-Fb, ERK was only transiently phosphorylated in response to IL-6 and was not involved in the antimitogenic effect of this cytokine (19). In the current study, inhibition of ERK activation did not prevent the proapoptotic effect of IL-6 on normal-Fb, suggesting that ERK activation does not play a significant role in IL-6–induced apoptosis. In support of this, Fukada and coworkers, using pro-B cell lines, showed that although gp130-dependent ERK signaling is required for proliferation, it had no effect on apoptosis (33). In contrast, ERK has been shown to inhibit apoptosis of a number of cell types including fibroblasts during anchorage or serum withdrawal (34), suggesting that it can induce antiapoptotic pathways. Indeed, in the current study, exposure of IPF-Fb to IL-6 led to an ERK-dependent increase in Bel-2 expression and a coordinate decrease in the expression of Bax. The possibility that ERK inhibits the STAT-3–dependent induction of a proapoptotic protein such as Bax is supported by the findings of Sengupta and colleagues, who demonstrated that IL-6–induced activation of STAT-3 in myeloid cells is inhibited by ERK (35). Therefore, activation of the ERK signaling pathway by binding of IL-6 to its receptor appears to play a significant role in inhibition of apoptosis in IPF-Fb, and a possible mechanism may involve inhibition of STAT-3–dependent Bax expression and a coordinate increased expression of Bel-2.

Our findings that in normal-Fb, the proapoptotic effects of IL-6 are completely dependent on STAT-3 are at odds with previous work showing that constitutively active STAT-3 protects murine fibroblasts from ultraviolet radiation and serum withdrawal (34). The reasons for this discrepancy are not clear, but may relate to our use of a single cytokine (IL-6) to activate gp130 compared with chronic, cytokine-independent activation of STAT-3 in the model used by Shen and coworkers (34). Given that gp130 is the common signal transducing subunit for a family of cytokines, the final effect might depend on which cytokine binds and activates the receptor complex (32). Indeed, our finding that IL-11 is antiapoptotic in normal-Fb supports this hypothesis.
As is the case with IL-6, little is known about the effects of IL-11 on fibroblast apoptosis. Interestingly, mice overexpressing IL-11 in the lung demonstrate an increased number of myofibroblasts, suggesting that IL-11 may inhibit apoptosis in these cells. This is supported by our results demonstrating that IL-11 protects against Fas-induced apoptosis in both normal-Fb and IPF-Fb. Furthermore, the probable mechanism of this effect appears to involve a STAT-5-dependent increase in Bcl-2 expression, resulting in an increased Bcl-2/Bax ratio.

In conclusion, we have demonstrated a fundamental abnormality involving IL-6 signaling through gp130 in IPF-Fb. Specifically, IL-6 protects against Fas-induced apoptosis in IPF-Fb, whereas it enhances the apoptotic effect of Fas in normal-Fb. The contrasting effects of IL-6 in IPF-Fb and normal-Fb appear to be associated with differential activation of STAT-3 and ERK. In normal-Fb, IL-6 signaling through STAT-3 appears to predominate, resulting in increased expression of Bax and enhancement of Fas-induced apoptosis. In IPF-Fb, IL-6 signaling through ERK is upregulated, with consequent inhibition of STAT-3-mediated Bax expression and an increase in Bcl-2 expression. Although the reasons why IL-6 signaling through ERK is upregulated in IPF-Fb are not known, these findings complement our previous work showing that IL-6 inhibited proliferation of normal-Fb by activating STAT-3, but enhanced proliferation IPF-Fb through an ERK-dependent mechanism (19). Taken together, the data suggests that under normal conditions IL-6 does not initiate and may protect against fibrosis, by enhancing fibroblast apoptosis. However, in diseases such as IPF in which fibroblasts appear to be phenotypically different, it seems likely that IL-6 significantly contributes to the development of fibrosis.

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