Diminished Prostaglandin E_2 Contributes to the Apoptosis Paradox in Idiopathic Pulmonary Fibrosis

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Rationale: **Patients with idiopathic pulmonary fibrosis (IPF), a progressive disease with a dismal prognosis, exhibit an unexplained disparity of increased alveolar epithelial cell (AEC) apoptosis but reduced fibroblast apoptosis.**

Objectives: **To examine whether the failure of patients with IPF to up-regulate cyclooxygenase (COX)-2, and thus the antifibrotic mediator prostaglandin (PG)E_2, accounts for this imbalance.**

Methods: **Fibroblasts and primary type II AECs were isolated from control and fibrotic human lung tissue. The effects of COX-2 inhibition and exogenous PGE_2 on fibroblast and AEC sensitivity to Fas ligand (FasL)-induced apoptosis were assessed.**

Measurements and Main Results: **IPF lung fibroblasts are resistant to FasL-induced apoptosis compared with control lung fibroblasts. Inhibition of COX-2 in control lung fibroblasts resulted in an apoptosis-resistant phenotype. Administration of PGE_2 almost doubled the rate of FasL-induced apoptosis in fibrotic lung fibroblasts compared with FasL alone. Conversely, in primary fibrotic lung type II AECs, PGE_2 protected against FasL-induced apoptosis. In human control and, to a greater extent, fibrotic lung fibroblasts, PGE_2 inhibits the phosphorylation of Akt, suggesting that regulation of this prosurvival protein kinase is an important mechanism by which PGE_2 modulates cellular apoptotic responses.**

Conclusions: **The observation that PGE_2 deficiency results in increased AEC but reduced fibroblast sensitivity to apoptosis provides a novel pathogenic insight into the mechanisms driving persistent fibroproliferation in IPF.**

Keywords: alveolar epithelial cell; fibroblast; pathogenesis; Fas ligand; Akt

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Excess epithelial cell apoptosis and fibroblast resistance to apoptosis are believed to contribute to the fibroproliferation that characterizes idiopathic pulmonary fibrosis (IPF). The mechanism underlying this imbalance in apoptosis is unknown.

What This Study Adds to the Field

The diminished capacity of patients with IPF to produce prostaglandin (PG)E_2 results in increased sensitivity of alveolar epithelial cells to Fas ligand–induced apoptosis but induces fibroblast resistance to the same stimulus.

Idiopathic pulmonary fibrosis (IPF) is postulated to develop as a consequence of an aberrant wound healing response after recurrent alveolar injury (1, 2). Prostaglandin (PG)E_2, the major prostanoid in the lung, is an important antifibrotic lipid mediator (3–5). PGE_2 is found in reduced levels in the lungs of patients with IPF (6). This reduction, due to a failure of patients with IPF to up-regulate the expression of the key inducible enzyme in the PGE_2 biosynthetic pathway cyclooxygenase (COX)-2, has been demonstrated to contribute to increased proliferation and collagen production by fibroblasts from IPF lung after transforming growth factor (TGF)-β stimulation when compared with control lung fibroblasts (4, 7). In the murine bleomycin model of pulmonary fibrosis, COX-2–deficient animals develop worse fibrosis than wild-type littermates (4, 8).

Apoptosis is important in the resolution phase of the normal wound healing response (9). In healthy peripheral human lung only sporadic cells undergo apoptosis. In IPF, however, widespread epithelial apoptosis is observed (10–12). In contrast to epithelial cells, fibroblasts derived from IPF lung are more resistant to apoptosis than control lung fibroblasts (13–16). A number of mechanisms have been proposed for this imbalance in fibroblast and epithelial cell apoptosis. Fibroblasts from IPF lungs induce alveolar epithelial cell (AEC) apoptosis *in vitro*, at least in part through the paracrine secretion of angiotensin II (17). Resistance of fibroblasts to apoptosis in IPF has been suggested to be due, in part, to altered responses to IL-6 (14). A number of other mediators, including TGF-β, tumor necrosis factor (TNF)-α, IL-1β and FIZZ1 (found in inflammatory zone) have been demonstrated to play a role in modulating either fibroblast or AEC apoptosis (18–22). However, none of these mechanisms has been shown in humans to explain the increased epithelial cell but decreased fibroblast apoptosis observed in IPF.

Outside the lung, COX-2 and PGE_2 play integral roles in regulating the apoptosis of both epithelial cells and fibroblasts. COX-2 overexpression plays an important part in the development of a range of epithelial-derived tumors through inhibition of epithelial apoptosis (23). In a murine model of radiation-induced gastric epithelial injury, exogenous PGE_2 protects epithelial cells from apoptosis (24). In contrast, apoptosis of both human synovial fibroblasts and colonic fibroblasts is promoted by PGE_2 (25, 26).

This study tested the hypothesis that the limited expression of COX-2 and PGE_2 observed in patients with IPF contributes to both enhanced epithelial apoptosis and reduced fibroblast apoptosis. Our results demonstrate that PGE_2 promotes AEC survival but sensitizes fibroblasts to Fas ligand (FasL)-induced apoptosis via a mechanism involving phosphorylation of Akt.

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Furthermore, we have shown that in patients with IPF, deficient PGE_2 production results, at least in part, in increased AEC but reduced fibroblast apoptosis. Some of the data in this paper have previously been reported in abstract form (27, 28).

METHODS

Patient Population

Fibrotic lung tissue was obtained at transplant surgery (n = 3, aged 52 ± 10.6 yr, two men, two IPF, one scleroderma fibrotic nonspecific interstitial pneumonia [29]) and from patients undergoing surgical lung biopsy (n = 6, aged 60.8 ± 20.0 yr, two men, six IPF). Control lung tissue was obtained from histologically normal areas of peripheral lung removed at lung cancer resection (n = 6, aged 52 ± 17.3 yr, three men). Additional samples of paraffin-embedded lung tissue were from the pathology archive of the Royal Brompton Hospital (10 IPF, aged 57.6 ± 3.6 yr and 6 control subjects, aged 58.2 ± 7.6 yr). All tissue was obtained with appropriate consent and its use approved by the relevant local research ethics committee.

Tissue Culture

Primary human lung fibroblasts were isolated as previously described (4). Type II AECs were isolated from lung tissue obtained at lung transplantation using the technique described by Thorley and colleagues (30). Additional experiments were performed using the A549 type II AEC cell line. Further information is provided in the online supplement.

Induction and Detection of Apoptosis

Fibroblasts and AECs were incubated as necessary with COX inhibitors or exogenous PGE_2 for 16 hours before being exposed to FasL (Calbiochem, CA) (50 ng/ml for 24 h unless otherwise stated). Experimental agents were added at doses and times indicated in the figure legends and text, and are described in more detail in the online supplement. Apoptosis was detected by Annexin V/propidium iodide staining and analyzed by flow cytometry. Verification of results was performed by morphological assessment of cell nuclei as described by Uhal and colleagues (31). These methods are described in more detail in the online supplement.

Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded sections of human lung tissue using the avidin-biotin antibody complexing method, as previously described (32).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Roche TUNEL kit; Roche Applied Science, Burgess Hill, UK) was performed on paraffin sections according to the manufacturer's instructions. Binding of labeled nucleotides was visualized with NBT/BCIP solution (Roche). Sections were counterstained with nuclear fast red.

PGE_2 Quantification

PGE_2 quantification was performed, according to manufacturer's instructions, using a Biotrak Enzymeimmunoassay (GE Healthcare, Amersham, UK).

Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot were performed as previously described (8) with further detail in the online supplement.

Statistics

Statistical analyses were performed using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA). Data for individual experiments are reported as mean ± SEM. Group data are presented as the median. Parametric data were tested using either a Student *t* test or analysis of variance with Tukey multiple comparison test. Nonparametric data were analyzed using a Mann-Whitney *U* test.

RESULTS

Apoptosis of Epithelial Cells but Not Fibroblasts Is Frequently Observed in IPF Lung Tissue

To assess apoptosis *in vivo* in IPF, paraffin-embedded sections of lung tissue were TUNEL stained and immunohistochemically stained for active caspase 3 and cleaved poly(ADP-ribose) polymerase (Figure 1). All three methods are considered to demonstrate cells that have terminally activated the apoptotic cascade and are in the process of undergoing apoptosis. In keeping with previous studies that have used either TUNEL or active caspase 3 staining (10, 11, 13) we found evidence, by all three methods, of frequent epithelial cell apoptosis. Epithelial apoptosis was seen most often in regions of hyperplastic epithelium, especially in areas overlying fibroblastic foci, and was also seen in bronchial epithelium and in areas of apparently normal alveolar epithelium. By contrast, fibroblast-like cells and cells within regions of fibrosis were rarely seen to be apoptotic. In normal lung sections apoptosis was very rarely seen, with there often being no apoptotic cells detectable across whole tissue sections (data not shown).

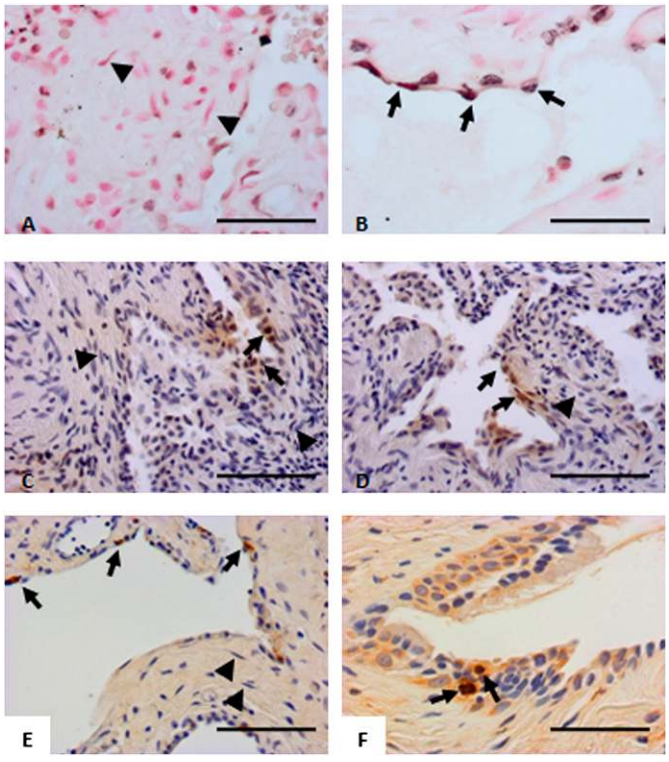

Figure 1. Excess epithelial apoptosis in idiopathic pulmonary fibrosis. Photomicrographs of idiopathic pulmonary fibrosis (IPF) lung after (*A, B*) terminal deoxynucleotidyl transferase dUTP nick end labeling staining and (*C, D*) immunohistochemical staining for active caspase 3 and (*E, F*) cleaved poly(ADP-ribose) polymerase. All three methods demonstrate frequent epithelial apoptosis (*arrows*) most frequently occurring in hyperplastic epithelial cells, especially those overlying fibroblastic foci (*B*). By contrast, fibroblast-like cells showed no evidence of apoptosis (*arrowheads*). Images are representative of staining performed on samples from 10 individuals with IPF. Scale bar represents 100 μm.

IPF Lung Fibroblasts Are Resistant to FasL-induced Apoptosis When Compared with Control Lung Fibroblasts

FasL, through binding of Fas (CD95), is a potent activator of the extrinsic apoptotic pathway. Both control and fibrotic lung fibroblasts have been demonstrated *in vitro* to express membrane-bound Fas (16). Preliminary experiments confirmed that human recombinant FasL induces apoptosis of primary human lung fibroblasts in a concentration- and time-dependent manner (*see* online data supplement for more detail). We and others have previously reported that fibroblasts from fibrotic lung are resistant to FasL-induced apoptosis when compared with fibroblasts from control lung (14–16). To confirm this was the case for this current collection of primary fibroblast lines, we exposed cells to 50 ng/ml FasL for 24 hours and then measured apoptosis by annexin V/propidium iodide staining and fluorescence-activated cell sorter (FACS) analysis (Figure 2). Fibroblasts from six control patients and nine patients with pulmonary fibrosis were assessed. The median increase in FasL-induced apoptosis compared with untreated cells for fibrotic lung fibroblasts was more than fivefold lower than for control lung fibroblasts ($P < 0.005$).

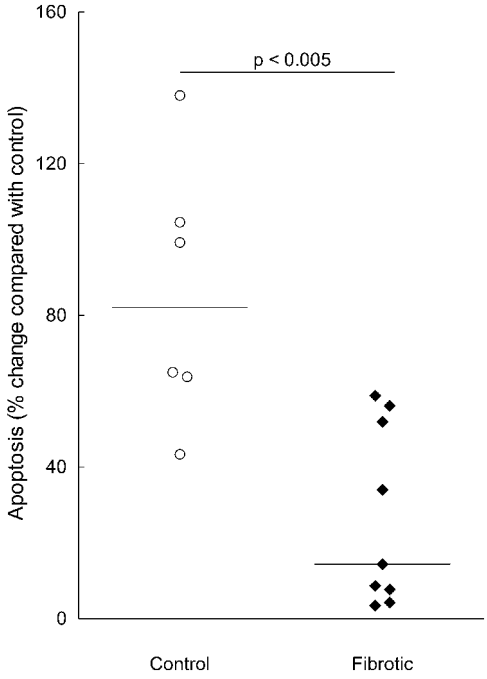

Figure 2. Fibrotic lung fibroblasts are resistant to Fas ligand (FasL)-induced apoptosis. Differences in apoptotic response to FasL (50 ng/ml for 24 h) were assessed in six separate lines of primary control (*circles*) lung fibroblasts and nine lines of fibrotic (*diamonds*) primary lung fibroblasts. Apoptosis was measured by annexin V/propidium iodide staining and fluorescence-activated cell sorter analysis. Each point represents an individual cell line. The bar represents the median value for each group.

COX-2 Inhibition Increases Control Lung Fibroblast Resistance to FasL-induced Apoptosis

To assess the role of COX-2 in modulating the apoptotic response of fibroblasts to FasL-induced apoptosis, control lung fibroblasts were incubated with the nonspecific COX-1/-2 inhibitor indomethacin (1μg/ml) or the selective COX-2 inhibitor NS398 (5 μg/ml) before exposure to FasL. Apoptosis was assessed morphologically by fluorescence microscopy after propidium iodide staining of fixed cells and by annexin V/propidium iodide staining and FACS analysis (Figure 3). COX-2 inhibition significantly increased control lung fibroblast resistance to FasL-induced apoptosis but did not affect apoptosis in the absence of FasL. When assessed morphologically in a representative control fibroblast line (Figures 3A–3C), apoptosis decreased from 55.3 ± 9.0% to 29.8 ± 9.35% in cells incubated with indomethacin ($P < 0.001$) and to 23.8 ± 3.2% for cells incubated with NS398 ($P < 0.001$ compared with control; nonsignificant compared with indomethacin). Five separate lines of primary control lung fibroblasts were tested by FACS analysis. For each of these lines, COX-2 inhibition with either indomethacin or NS398 increased resistance to FasL-induced apoptosis (Figure 3D). The median percentage change compared with control in FasL-induced apoptosis for the five lines was 63.8%; this decreased to 24.4% in the presence of indomethacin ($P < 0.01$ compared with control) and 31.8% with NS398 ($P < 0.001$ compared with control). There was no significant difference in FasL-induced apoptosis between indomethacin- and NS398-exposed fibroblasts.

PGE_2 Increases the Sensitivity of Fibrotic Lung Fibroblasts to FasL-induced Apoptosis

Fibrotic fibroblasts, as noted, are resistant to FasL-induced apoptosis. As illustrated in data from a representative fibroblast cell line, COX-2 inhibition with either indomethacin or NS398 had no further effect on this resistance (Figure 4A). Administration of exogenous PGE_2 at a dose of 10^{-7} M significantly increased the sensitivity of the same fibroblast line to FasL by approximately 2.5-fold ($P < 0.001$). Exogenous PGE_2 had no significant effect on apoptosis in fibroblasts not exposed to FasL. Phase contrast microscopy of fibroblasts incubated with FasL demonstrated a reduction in cell number compared with untreated cells and clear morphological evidence of apoptosis (Figures 4B−4C). Many of the fibroblasts treated with FasL were shrunken or rounded up. Detached fibroblasts floating in the media were observed in the FasL-exposed but not the untreated control cells. Exogenous PGE_2 in addition to FasL dramatically increased apoptosis, resulting in a clearly discernible increase in the number of both shrunken, rounded-up fibroblasts and of detached fibroblasts (Figure 4D). Overall, PGE_2 increased the rate of FasL-induced apoptosis in each of six lines of fibrotic lung fibroblasts tested (Figure 4E). The fibroblasts from scleroderma lung did not differ from the IPF lung fibroblasts in either their resistance to apoptosis or their response to exogenous PGE_2. The median rate of apoptosis for fibrotic lung fibroblasts exposed to FasL alone was 8.2%. This increased to 34.9% after PGE_2 administration ($P < 0.01$). The PGE_2-mediated increase in FasL-induced apoptosis in fibrotic lung fibroblasts, as measured in a representative cell line, was concentration dependent (Figure 5A). The rate of apoptosis increased from 52.7 ± 11.2% to 78.5 ± 19.9% after incubation with 10^{-7} M PGE_2 ($P < 0.05$) and increased further to 102.0 ± 14.7% with 10^{-5} M PGE_2 ($P < 0.001$). Fibrotic lung fibroblasts exposed to PGE_2 without FasL did not show any increase in apoptosis even at the maximum PGE_2 concentration tested of 10^{-5} M (Figure 5A). By contrast to fibrotic lung fibroblasts, fibroblasts from control lung showed no significant change in the rate of FasL-induced apoptosis at any of the concentrations of PGE_2 tested (Figure 5B). However, when control fibroblast COX-2 was inhibited by pretreatment for 16 hours with indomethacin (1 μg/ml), exogenous PGE_2 resulted in a concentration-dependent increase in FasL-induced apoptosis (Figure 5C).

FasL Induces Fibroblast PGE_2 Production

PGE_2 levels in media were measured after 24 hours of exposure of fibroblasts to FasL (50 ng/ml). Interestingly, FasL stimulated

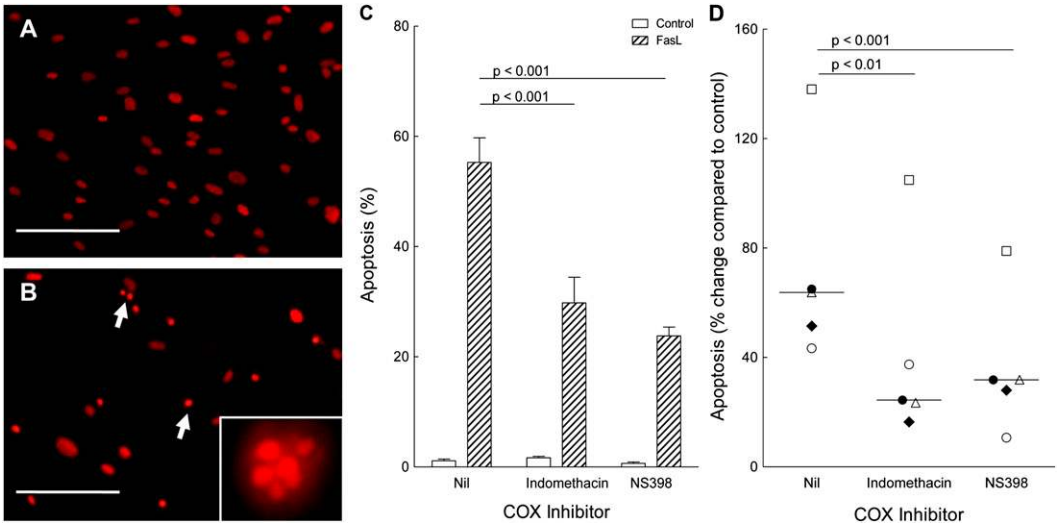

Figure 3. Cyclooxygenase (COX)-2 inhibition protects control lung fibroblasts from Fas ligand (FasL)-induced apoptosis. Apoptosis was assessed morphologically in fixed untreated cells that were permeabilized and stained with propidium iodide. (*A*) Untreated fibroblasts, (*B*) FasL-treated fibroblasts. Apoptotic nuclei initially demonstrate condensation of chromatin (*B*, high-power inset) before progressing to form dense, highly fluorescent apoptotic bodies (*arrows* in *B*). (*C*) Control lung fibroblasts were cultured overnight in serum-free media alone or with the nonselective COX inhibitor indomethacin (1 μg/ml), or the selective COX-2 inhibitor NS398 (5 μg/ml). Fibroblasts were then maintained for 24 hours in serum-free media alone or with COX inhibitors or were exposed to FasL ± COX inhibitors. Apoptosis was determined morphologically by counting apoptotic and nonapoptotic cells in five high-power fields for each experimental replicate. Each bar represents the mean ± SEM of four replicates. Five individual lines of control lung fibroblast were similarly treated with FasL (50 ng/ml) alone or with indomethacin or NS398. (*D*) Apoptosis was measured by annexin V/propidium iodide staining and flow cytometry. Each point represents an individual cell line with unique symbols to enable comparison between different culture conditions. The median for each experimental condition is shown.

the production of PGE_2 by fibroblasts. PGE_2 levels in media from untreated control lung fibroblasts were 328.2 ± 83.68 pg/ml (n = 6) and this increased significantly to 1,256 ± 580.2 pg/ml after FasL ($P < 0.0001$). For fibrotic lung fibroblasts, untreated PGE_2 levels were 143.4 ± 33.91 pg/ml (n = 9), and this also increased significantly after FasL to 271.8 ± 58.0 pg/ml ($P < 0.01$). The FasL-induced increase in PGE_2 levels was significantly greater in control as compared with fibrotic lung fibroblasts ($P < 0.05$). As expected, COX-2 inhibition with either indomethacin or NS398 significantly reduced the levels of PGE_2 detectable in cell culture media after FasL exposure. For control fibroblasts exposed to FasL, PGE_2 levels after indomethacin were 69.0 ± 34.8 pg/ml ($P < 0.0001$ compared with FasL alone) and with NS398 levels were 260.7 ± 123.6 pg/ml ($P <$

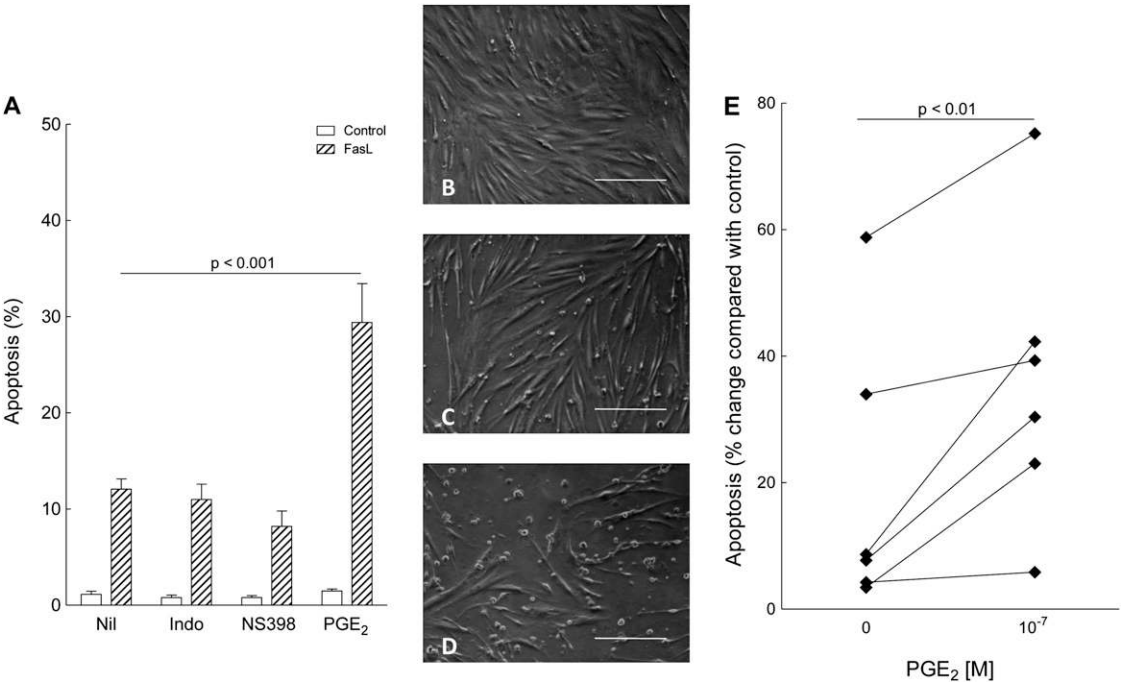

Figure 4. Exogenous prostaglandin (PG)E_2 increases fibrotic lung fibroblast apoptosis in response to Fas ligand (FasL). (*A*) Fibrotic lung fibroblasts were cultured overnight in serum-free media alone or with the nonselective cyclooxygenase (COX) inhibitor indomethacin (1 μg/ml) or the selective COX-2 inhibitor NS398 (5 μg/ml) or PGE_2 (10^{-7} M). Fibroblasts were then maintained for 24 hours in serum-free media ± COX inhibitors or PGE_2 alone or with FasL. Each bar represents the mean ± SEM of four replicates. (*B–D*) Phase contrast microscopy images of fibrotic lung fibroblasts grown in 6-well plates (*B*) in serum-free media alone, (*C*) with FasL, or (*D*) with FasL and 10^{-7} M PGE_2. (*B*) Untreated fibroblasts show a healthy elongated appearance. (*C*) After FasL exposure fibroblast number is reduced with evidence of rounded-up, detached apoptotic cells. (*D*) After the addition of exogenous PGE_2 the number of adherent healthy fibroblasts is dramatically reduced with a striking increase in apoptotic cells. (*E*) Six separate lines of fibrotic lung fibroblast were treated with FasL alone or in combination with PGE_2 and apoptosis measured by annexin V/propidium iodide staining and flow cytometry. Each point represents a mean of four replicates for each individual cell line.

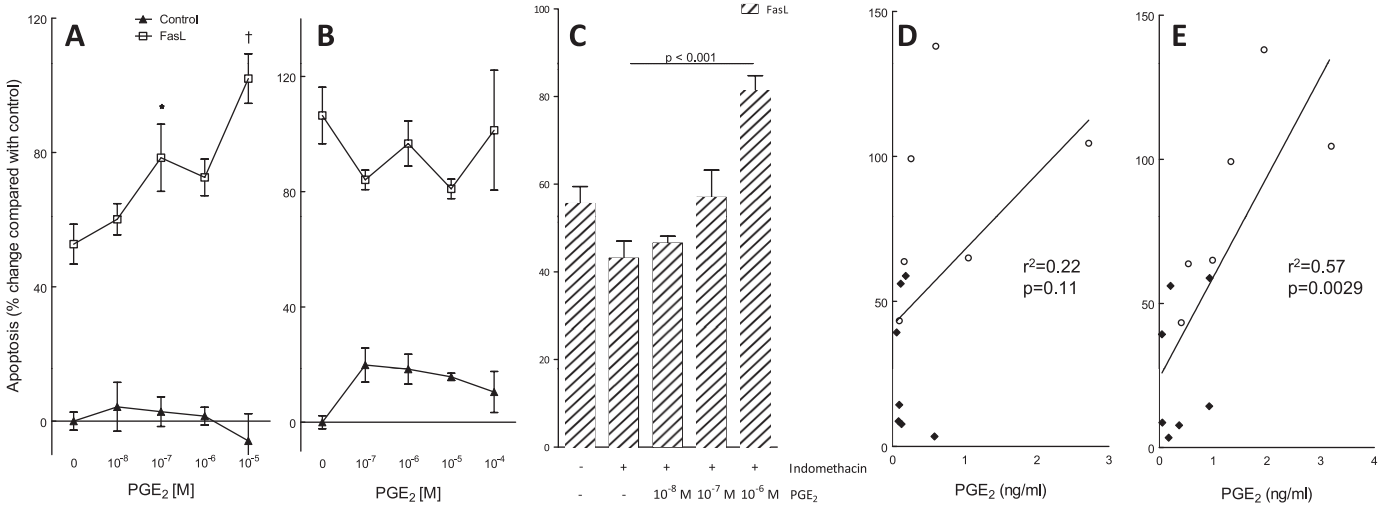

Figure 5. The relationship of prostaglandin (PG)E_2 to fibroblast Fas ligand (FasL)-induced apoptosis. Representative lines of (*A*) fibrotic and (*B*) control lung fibroblasts were treated with increasing log concentrations of PGE_2 in the presence or absence of FasL. (*C*) Control fibroblasts were additionally exposed to increasing log concentrations of PGE_2 after cyclooxygenase (COX) inhibition with indomethacin. Apoptosis was determined by annexin V/propidium iodide staining and flow cytometry. The data are expressed as change in apoptosis compared with baseline level of apoptosis measured in untreated cells. (*A*) For fibrotic lung fibroblasts this was 9.55 ± 0.053% and (*B*) for control lung fibroblast it was 7.27 ± 0.50% and (*C*) 9.41 ± 0.81%. (*D*, *E*) Rates of apoptosis for individual lines of fibroblasts. Six control fibroblast lines (*circles*) and nine fibrotic lung fibroblast lines (*diamonds*) as shown in Figure 2 after FasL exposure (Y axis) have been plotted against PGE_2 values measured in the cell culture supernatant of the same fibroblast lines grown in media containing (*D*) 0.2% serum alone or (*E*) after transforming growth factor-β (1 ng/ml) stimulation. The degree of correlation between apoptosis and individual cell lines PGE_2 synthetic capacity is demonstrated by a line of best fit as determined by linear regression.

0.0001). The corresponding PGE_2 levels for fibrotic fibroblasts were: indomethacin 14.4 ± 8.1 pg/ml ($P < 0.0001$) and NS398 84.4 ± 22.7 ($P < 0.001$).

The Sensitivity of Fibroblasts to FasL-induced Apoptosis Correlates with Their Capacity to Induce COX-2 and Thus PGE_2 Synthesis

In previous work we have shown that fibroblasts from control lung induce COX-2 expression and thus PGE_2 synthesis in response to TGF-β stimulation (4). By contrast, fibrotic lung fibroblasts exhibit a reduced capacity to increase COX-2 expression in response to TGF-β. The capacity for individual fibroblast lines to induce PGE_2 synthesis was assessed by measuring PGE_2 concentrations in the culture media of cells exposed to 1 ng/ml of TGF-β in media containing 0.2% FCS for 24 hours. The sensitivity of fibroblasts to FasL-induced apoptosis, as determined by increase in apoptosis compared with untreated cells after FACs analysis of annexin V/propidium iodide–stained cells, was compared with baseline and TGF-β–stimulated levels of PGE_2. There was no correlation between fibroblast apoptosis and basal PGE_2 levels (Figure 5D). However, there was a marked positive correlation between TGF-β–induced PGE_2 levels and FasL-induced fibroblast apoptosis ($r^2 = 0.5699$, $P = 0.0029$) (Figure 5E). Fibroblast FasL-induced PGE_2 levels correlated with TGF-β–induced induction of PGE_2 expression ($r^2 = 0.34$, $P = 0.038$). TGF-β–induced levels of PGE_2 are presented in preference to FasL-induced levels of PGE_2 as we believe that these more accurately represent the cells' capacity to produce PGE_2. FasL-induced levels of PGE_2 are likely to be less comparable between cell lines because of varying degrees of cell death and thus PGE_2 production.

PGE_2 Protects AECs from Fas-induced Apoptosis

Experiments to assess the role of COX-2 and PGE_2 in modulating the apoptotic response of AECs to FasL were performed using the A549 immortalized human type II AEC cell line with verification of key findings performed in primary type II AECs isolated from fibrotic lung tissue. A549 cells were sensitive to FasL (Figure 6A) with apoptosis increasing by 69.9 ± 6.6% compared with media control ($P < 0.01$). COX-2 inhibition with either indomethacin or NS398 resulted in a significant increase in apoptosis in response to FasL when compared with FasL exposure alone (both $P < 0.001$). COX-2 inhibition in the absence of FasL had no effect on AEC apoptosis. Administration of PGE_2 to A549 cells exposed to indomethacin and FasL resulted in a concentration-dependent reduction in apoptosis to levels observed with FasL alone (Figure 6B).

Primary type II AECs isolated from IPF lung were induced to undergo apoptosis by exposure to FasL (Figure 6C). Apoptosis was determined by annexin V/propidium iodide staining and FACS analysis. In contrast to fibroblasts derived from the same patient, type II AECs readily underwent FasL-induced apoptosis, with apoptosis increasing by 61.8 ± 3.2% compared with media control ($P < 0.001$). The addition of exogenous PGE_2 partially protected the fibrotic lung AECs from FasL-induced apoptosis, reducing the rate of apoptosis by 29.2% compared with FasL treatment alone ($P < 0.01$).

A549 cells produced 118.5 ± 7.1 pg/ml PGE_2 at baseline and 142.8 ± 13.1 pg/ml after FasL ($P < 0.05$ compared with baseline). Both indomethacin (48.29 ± 7.29 pg/ml, $P < 0.001$ compared with FasL alone) and NS398 (25.08 ± 1.34 pg/m, $P < 0.001$ compared with FasL alone) effectively inhibited PGE_2 production by FasL-exposed A549 cells. Primary type II AECS produced 170.3 ± 29.3 pg/ml of PGE_2 basally and 139.0 ± 13.9 pg/ml after FasL exposure (nonsignificant compared with basal levels). Because of the limited numbers of primary type II AECs available it was not possible to assess the effects of COX-2 inhibition on FasL-induced apoptosis.

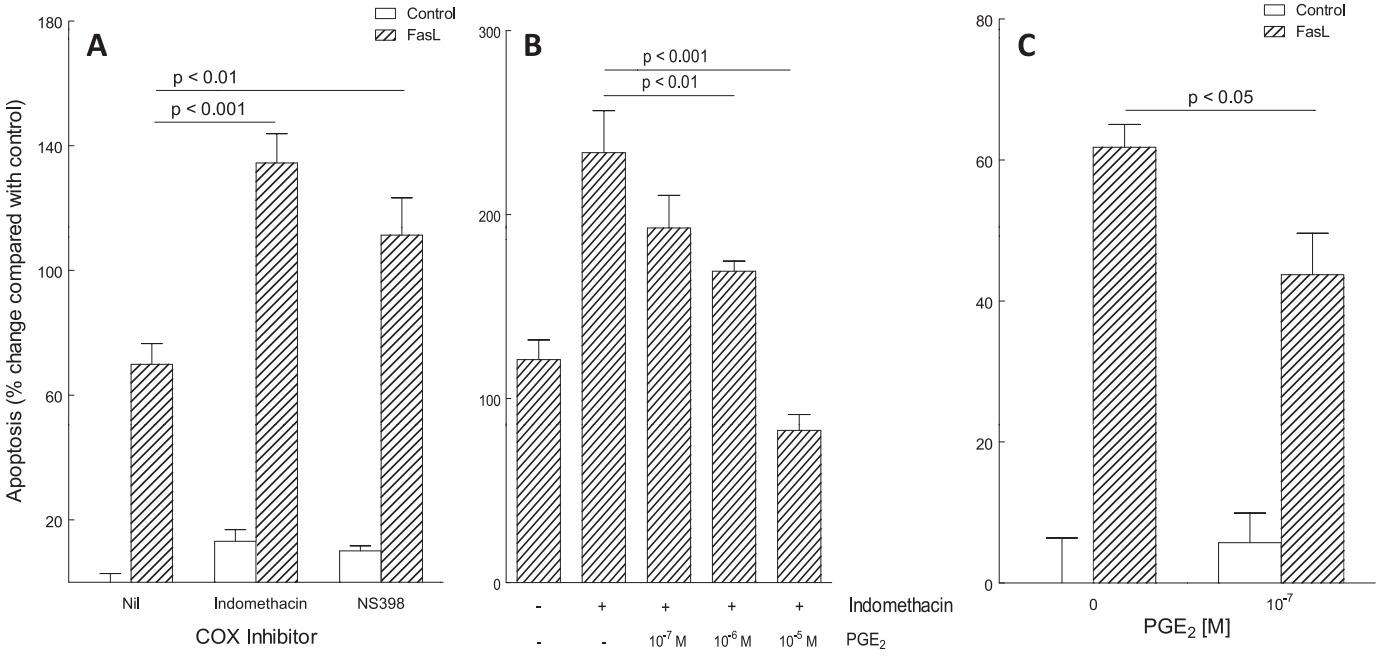

Figure 6. Role of prostaglandin (PG)E_2 in regulating Fas ligand (FasL)-induced type II alveolar epithelial cell (AEC) apoptosis. (*A*) The type II AEC cell line A549 were incubated in serum-free media alone or with indomethacin or NS398 overnight before addition of FasL. Apoptosis was determined by annexin V/propidium iodide staining. Data are expressed as change in apoptosis compared with untreated cells (3.42 ± 0.10%). (*B*) A549 cells were incubated with FasL alone or together with indomethacin (1 μg/ml) before and during exposure to increasing log concentrations of exogenous PGE_2. Apoptosis was determined morphologically by counting five high-power fields in each of four replicates per treatment condition. Bars represent the mean ± SEM of these counts. (*C*) Primary type II AECs derived from fibrotic lung tissue were exposed for 24 hours to FasL ± PGE_2 (10^{-7} M) and apoptosis determined by annexin V/propidium iodide and flow cytometry. Data are expressed as change in apoptosis compared with untreated cells (11.96 ± 0.77%). Bars represent mean ± SEM of four to six replicates per treatment condition.

COX-2/PGE_2 Modulate the Phosphorylation of the Prosurvival Protein Kinase Akt

Akt (protein kinase B) is a serine/threonine kinase that plays a central role in regulating a variety of signal transduction pathways involved in cell proliferation and apoptosis. Activation of Akt occurs through phosphorylation, a process that may be triggered by a variety of growth factors. To assess the effect of PGE_2 on Akt phosphorylation, control and fibrotic lung fibroblasts were serum starved overnight before incubation with either FasL alone or in combination with PGE_2. A549 AECs were incubated with either FasL alone or in combination with indomethacin. Cells were lysed in RIPA buffer at time intervals between 30 minutes and 12 hours after FasL exposure. As determined by Western blotting, Akt is only partially phosphorylated in basal untreated control and fibrotic fibroblasts (Figures 7A and 7C). PGE_2 almost totally abolishes Akt phosphorylation in both control and fibrotic lung fibroblasts (Figures 7B and 7C). In both control and fibrotic lung fibroblasts the degree of Akt phosphorylation increases dramatically within 30 minutes of exposure to FasL. PGE_2, however, markedly attenuates FasL-induced phosphorylation of Akt. PGE_2-induced attenuation of Akt phosphorylation was most prominent at 30 minutes and 1 hour after FasL treatment but persisted over the whole 12-hour time course. The magnitude of change in Akt phosphorylation after FasL exposure and thus the relative effect of PGE_2 in inhibiting this phosphorylation was markedly higher in fibrotic as compared with control lung fibroblasts (Figure 7D). In keeping with the presented data on the effect of PGE_2 on FasL-induced fibroblast apoptosis, levels of cleaved caspase 3 were higher in PGE_2-exposed cells at both 6 and 12 hours after FasL treatment (Figure 7A). Western blotting for phosphorylated AKT (p-Akt) in A549 cells demonstrates that basal levels of p-Akt are high but gradually diminish at 1 through 12 hours after FasL exposure (Figure 7E). The level of p-Akt, however, returns to baseline in A549 cells by 24 hours after FasL exposure. The phosphorylation of Akt is not affected in A549s by COX-2 inhibition.

PGE_2 Down-Regulates X-linked Inhibitor of Apoptosis Protein in Fibroblasts

X-linked inhibitor of apoptosis protein (XIAP) is the most potent apoptosis inhibitor in the inhibitor of apoptosis protein family. XIAP has been shown to be protected from breakdown by p-Akt–regulated phosphorylation (33). Both fibrotic and control lung fibroblasts express XIAP in the basal state (Figures 7A and 7C). After treatment with FasL, Western blotting for XIAP demonstrates that XIAP levels increase after 1 hour in fibroblasts exposed to FasL alone and that this increase is inhibited by the addition of PGE_2. In keeping with this finding, immunohistochemistry for XIAP shows no staining in control lung tissue (Figure 7F) but marked staining in IPF, with predominant localization to fibroblasts within both fibroblastic foci and within regions of dense mature fibrosis (Figures 7G and 7H). Epithelium in IPF shows only limited staining for XIAP.

DISCUSSION

In normal wound healing, the restitution of epithelial integrity and the apoptosis of fibroblasts/myofibroblasts are important steps in the resolution of injury and the restoration of tissue structure and function (1, 34). In IPF, excess epithelial apoptosis combined with fibroblast resistance to apoptosis appears to be an important mechanism underlying the development of progressive fibrosis (11, 13, 14). Our data

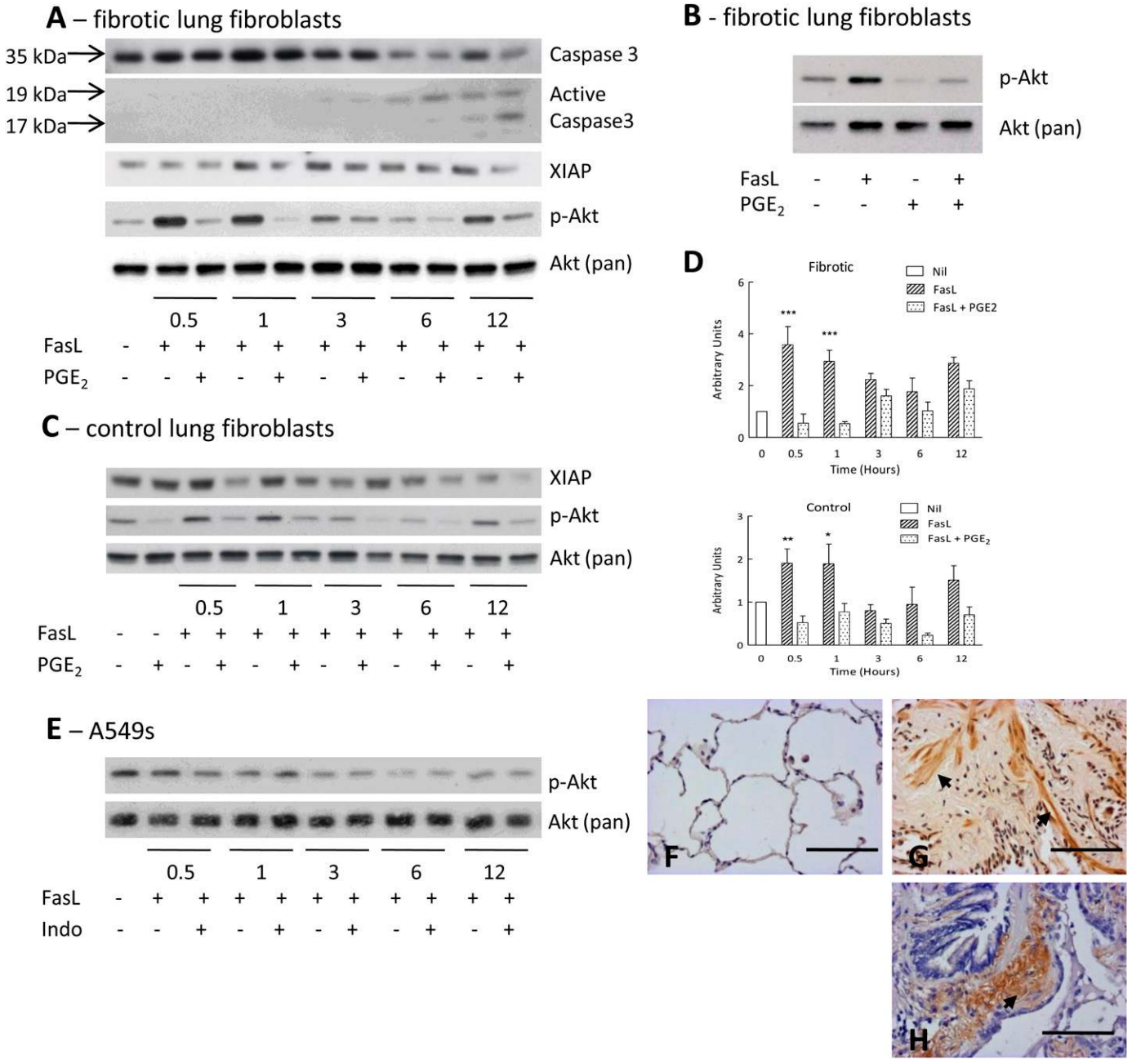

Figure 7. Cyclooxygenase-2/ prostaglandin (PG)E_2 modulate the phosphorylation of the prosurvival protein kinase Akt and expression of X-linked inhibitor of apoptosis protein (XIAP). (*A*) Fibrotic and (*C*) control lung fibroblasts were serum starved overnight before treatment with Fas ligand (FasL) with or without PGE_2 (10^{-7} M) for 24 hours. Cells were lysed in RIPA buffer at time points from 30 minutes through 12 hours after FasL administration. (*B*) In a separate experiment fibrotic lung fibroblasts were exposed to FasL for 24 hours before being lysed. (*E*) The A549 alveolar epithelial cell line was serum starved overnight before treatment with FasL with or without indomethacin (1 μg/ml). Western blots were then performed, as indicated, for total and cleaved caspase 3, XIAP, p-Akt, or total Akt. Representative images of three experiments performed with three replicate gels per experiment are shown. (*D*) Time-related changes in p-Akt in fibrotic (*upper panel*) and control (*lower panel*) lung fibroblasts were assessed semiquantitatively by image analysis. Results are normalized to p-Akt levels in basal unstimulated cells. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with basal p-Akt levels. Photomicrographs of (*F*) control and (*G* and *H*) idiopathic pulmonary fibrosis (IPF) lung immunohistochemically stained for XIAP demonstrate an absence of XIAP staining in healthy control lung tissue but localization of XIAP to fibroblast-like cells (*arrows*) within (*G*) established fibrosis and (*H*) fibroblastic foci in IPF. *Scale bar* represents 100 μm.

confirm the previously observed imbalance of increased epithelial but reduced fibroblast apoptosis in IPF. We have demonstrated that PGE_2 has opposing effects on the sensitivity of primary lung fibroblasts and alveolar epithelial cells to FasL-induced apoptosis. In fibrotic lung fibroblasts, we have shown that PGE_2 inhibits the phosphorylation of the prosurvival protein kinase Akt, suggesting that this is an important mechanism by which PGE_2 exerts its proapoptotic effect on fibroblasts. Furthermore, we have demonstrated, using patient-derived primary cells, that the failure of patients with IPF to up-regulate COX-2 and thus PGE_2 contributes to the observed paradox of reduced fibroblast

apoptosis but increased AEC apoptosis in the lungs of patients with IPF.

PGE_2 is a key antifibrotic mediator that plays an important role in wound resolution. Deficiency of PGE_2 in the lungs of patients with IPF has important profibrotic consequences (4, 8). PGE_2 has been shown outside the lung to play an important role in regulating the sensitivity of different cell types to apoptotic stimuli (23, 24). A recent study by Huang and colleagues demonstrates that PGE_2 induces fibroblast apoptosis and enhances the fibrotic response of fibroblasts to a combination of FasL and cycloheximide (35). Using both murine lung fibroblasts and the fetal lung fibroblast line IMR-90, Huang and colleagues were able to show that PGE_2 promotes apoptosis via a number of mechanisms, including decreased Akt phosphorylation, down-regulation of the prosurvival protein survivin, and up-regulation of Fas. Our observation that COX-2 inhibition increases the resistance of control lung fibroblasts to FasL-induced apoptosis and that exogenous PGE_2 restores the sensitivity of fibrotic lung fibroblasts to FasL-induced apoptosis is consistent with these findings and with previous observations made in synovial and colonic fibroblasts (25, 26). The variability in sensitization of different fibrotic lung fibroblast lines to FasL after exposure to PGE_2 is similar to the reported heterogeneous responses in fibroblast proliferation and collagen production induced by PGE_2 (36). In keeping with our findings in A549 cells, PGE_2 has previously been demonstrated to protect A549 cells from staurosporine-induced apoptosis (37). Our work is, as far as we are aware, novel in demonstrating that PGE_2 is protective against FasL-induced apoptosis in primary type II AECs derived from IPF lung. Because isolating primary AECs requires the availability of a significant quantity of lung tissue we have been limited in the experiments we have been able to perform in primary fibrotic lung AECs.

Apoptosis plays a crucial role in normal tissue homeostasis and is an important mechanism by which the body clears infected or terminally damaged cells. Dysregulated apoptosis has been shown to play a role in the development of a range of diseases, including many tumors, systemic lupus erythematosus, and Alzheimer disease (38, 39). In mice, induction of extensive alveolar epithelial apoptosis is sufficient, in itself, to induce pulmonary fibrosis (40). Conversely, bleomycin-induced fibrosis can be attenuated in mice by the administration of caspase inhibitors (41). Evidence in humans that aberrant apoptosis, in the form of excess AEC but reduced fibroblast apoptosis, is important in the evolution of IPF is, although circumstantial, compelling (11, 13, 14). A wide range of mediators, including IL-6, angiotensin II, TNF-α, and IL-1β (14, 17, 20, 22), have been shown to modulate the apoptotic response of a number of cell types in the lung; however, we believe that we are the first to demonstrate a single mediator that has differing effects on fibroblast and AEC apoptosis in human fibrotic lung. Another mediator that might also have opposing effects on fibroblast and AEC apoptosis in IPF is the profibrotic cytokine TGF-β. Although not investigated in IPF, TGF-β has been shown to increase resistance to apoptosis in rat lung fibroblasts, in the human fetal fibroblast line IMR-90, and in alveolar mesenchymal cells isolated from patients with acute lung injury (21, 42, 43). TGF-β has also been shown to sensitize human bronchiolar epithelial cells to Fas-induced apoptosis (18). TGF-β is an important stimulus for the up-regulation of COX-2, and thus PGE_2, by lung fibroblasts (4). Our data suggest that in the normal lung, after injury, PGE_2 opposes the effects of TGF-β, thus promoting AEC resistance to apoptosis and favoring fibroblast/myofibroblast apoptosis and by so doing promoting wound resolution. In IPF the failure of patients to up-regulate COX-2, combined with increased levels of TGF-β, may be sufficient to drive the paradox of fibroblast resistance to apoptosis and excess AEC apoptosis.

Our work has thrown up a number of as-yet unanswered questions. Interestingly, we have shown that FasL stimulates the production of PGE_2 by both control and fibrotic lung fibroblasts. This hitherto undescribed phenomenon may underlie the intrinsic differences in fibrotic and control lung fibroblast sensitivity to FasL-induced apoptosis. We have previously reported that fibrotic lung fibroblasts have a reduced capacity to up-regulate COX-2 and therefore PGE_2 in response to TGF-β stimulation (4). Although we have found that fibrotic fibroblasts increase PGE_2 release in response to FasL stimulation, the magnitude of increase is far less than that seen in control lung fibroblasts. Further to the observation of increased PGE_2 synthesis after FasL exposure, our data show that the sensitivity of individual fibroblast cell lines to FasL-induced apoptosis correlates with the capacity of the individual lines to induce PGE_2. It seems likely that this intrinsic difference in control and fibrotic lung fibroblasts explains our observation that fibrotic, but not control, lung fibroblasts showed a concen-

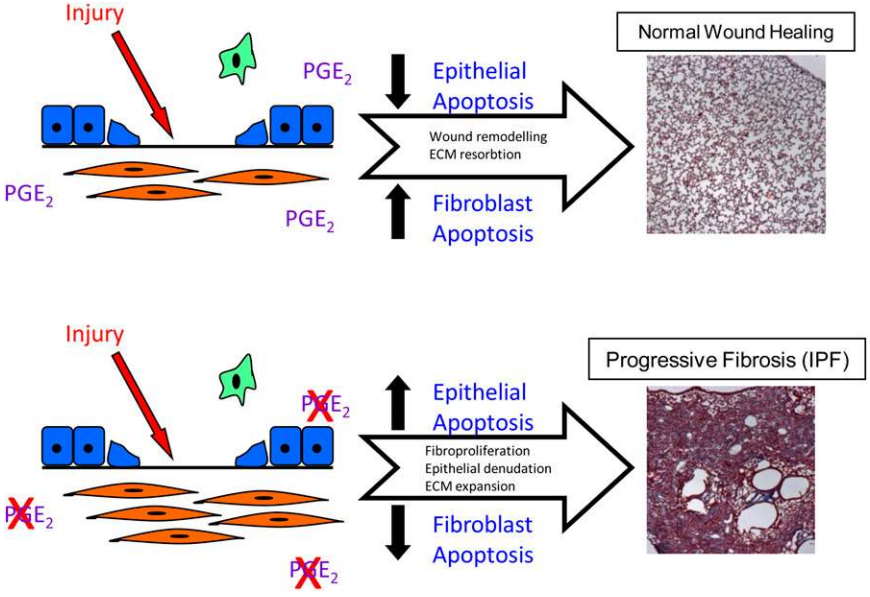

Figure 8. Schematic for proposed role of prostaglandin (PG)E_2 in idiopathic pulmonary fibrosis (IPF). After injury to the lung PGE_2 is produced by macrophages, airway epithelial cells, and fibroblasts. In healthy individuals (*top panel*) PGE_2 drives the resolution of scar tissue and does so in part by reducing epithelial apoptosis, thus facilitating restoration of epithelial integrity, and by promoting fibroblast apoptosis and thus clearance. This role of PGE_2 is part of a coordinated cascade of events leading to wound remodeling and restoration of tissue structure and function. In individuals with IPF there is a failure of induction of cyclooxygenase-2 and thus PGE_2. This increases the sensitivity of epithelial cells to apoptotic stimuli and promotes the survival and therefore persistence of fibroblasts/myofibroblast. The net effect of this deficiency of PGE_2 is to favor fibroproliferation and hence the progressive fibrosis that characterizes IPF.

tration-dependent increase in their sensitivity to FasL-induced apoptosis when exposed to exogenous PGE_2. Similarly, in fibrotic lung fibroblasts, cells that exhibit a reduced capacity to induce COX-2, COX-2 inhibition had relatively little additional effect on PGE_2 levels and also had no further effect on resistance to FasL-induced apoptosis. Interestingly, our data demonstrate that in both control and fibrotic lung fibroblasts, but not AECs, FasL acts as a trigger for the phosphorylation of the prosurvival kinase Akt. FasL stimulation of Fas has been previously reported to induce phosphorylation of Akt in murine epidermal cells (44).

The mechanisms by which FasL stimulates PGE_2 production and induces Akt phosphorylation are unclear. Fas is part of the TNF superfamily of receptors and TNF-α is known to promote COX-2 up-regulation (45). It is increasingly being recognized that Fas mediates not only apoptosis but also diverse nonapoptotic functions, depending on the tissue and the conditions under which the receptor is expressed (46). Our observation that fibrotic lung fibroblasts are both resistant to apoptosis and produce less PGE_2 in response to FasL than control fibroblasts suggests that alterations in both the apoptotic and nonapoptotic functions of Fas signaling are important in the pathogenesis of IPF.

Our data suggest that a mechanism by which PGE_2 increases the sensitivity of fibrotic lung fibroblast to FasL-induced apoptosis is by inhibiting phosphorylation, and thus activation, of the prosurvival kinase Akt. In our experiments, FasL-induced phosphorylation of Akt was greater in fibrotic than control lung fibroblasts. Consequently, the inhibitory effect of PGE_2 on Akt phosphorylation was magnified in fibrotic lung fibroblasts. Interestingly, in view of this observation, Xia and colleagues have recently shown that fibroblasts from fibrotic lung display aberrant activation of the phosphoinositide 3-kinase–Akt signal pathway when compared with control lung fibroblasts (47).

Because of the large amount of tissue required to isolate relatively few type II AECs from fibrotic lung, we have been limited in the number of experiments that we have been able to perform with these cells. We have therefore been unable to assess the effect of PGE_2 on Akt phosphorylation in primary AECs. The A549 cell line is immortalized; it is likely that this has an impact on the activation of prosurvival pathways in these cells. This may therefore explain the failure of COX-2 inhibition to alter Akt phosphorylation in these cells. In gut epithelium, however, PGE_2, acting on the EP2 and EP4 receptors, reduces radiation-induced apoptosis by increasing Akt phosphorylation (48). PGE_2 may have additional beneficial effects on AECs beyond inhibiting apoptosis. In kidney epithelial cells PGE_2 is a key inhibitor of epithelial to mesenchymal cell transition (EMT) (49). EMT has been shown to play an important pathogenetic role in the development of pulmonary fibrosis in mice (50). Further work is required to explore whether PGE_2 prevents AECs transforming into fibroblasts via EMT.

In conclusion, we have demonstrated that, in addition to its previously described actions, PGE_2 exerts an important antifibrotic effect by promoting the survival of AECs but increasing the sensitivity of fibroblasts/myofibroblasts to apoptosis. Furthermore, we provide evidence that in patients with IPF, deficiency of PGE_2 is, at least in part, responsible for the observed paradox of increased epithelial but reduced fibroblast apoptosis. These findings further substantiate the notion that the consequence of reduced PGE_2 in the lungs of patients with IPF is to perpetuate fibroproliferation (Figure 8). Our results provide further support for the idea that IPF arises as a result of an aberrant wound healing response in the lung that is characterized by an imbalance between pro- and antifibrotic mediators. Overall, our findings suggest that PGE_2-mediated signaling pathways offer a potentially attractive target for therapeutic drug development in IPF.

Conflict of Interest Statement: T.M.M. received $1,001–$5,000 from GlaxoSmithKline, $1,001–$5,000 from Actelion, and $1,001–$5,000 from Phillips Respironics in consultancy fees and more than $100,001 from GlaxoSmithKline in an unrestricted research grant. I.C.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.E.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.F.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.J.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.G.N. received $10,001–$50,000 from Boehringer Ingelheim Ltd, $10,001–$50,000 from Actelion Ltd, and $1,001–$5,000 from AstraZeneca Ltd in consultancy fees. G.J.L. received $1,001–$5,000 from Centocor in consultancy fees. T.D.T. received more than $100,001 from Unilever in institutional grants. R.C.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.J.M. received $10,001–$50,000 from GlaxoSmithKline in consultancy fees, $5,001–$10,000 from AstraZeneca for serving as an expert witness, and more than $100,001 from GlaxoSmithKline in collaborative grants.

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