Endothelin-1 and Transforming Growth Factor- β 1 Independently Induce Fibroblast Resistance to Apoptosis via AKT Activation

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Myofibroblast apoptosis is critical for the normal resolution of wound repair responses, and impaired myofibroblast apoptosis is associated with tissue fibrosis. Lung expression of endothelin (ET)-1, a soluble peptide implicated in fibrogenesis, is increased in murine models of pulmonary fibrosis and in the lungs of humans with pulmonary fibrosis. Mechanistically, ET-1 has been shown to induce fibroblast proliferation, differentiation, contraction, and collagen synthesis. In this study, we examined the role ET-1 in the regulation of lung fibroblast survival and apoptosis. ET-1 rapidly activates the prosurvival phosphatidylinositol 3'-OH kinase (PI3K)/AKT signaling pathway in normal and fibrotic human lung fibroblasts. ET-1-induced activation of PI3K/AKT is dependent on p38 mitogen-activated protein kinase (MAPK), but not extracellular signal-regulated kinase (ERK) 1/2, JNK, or transforming growth factor (TGF)- β 1. Activation of the PI3K/AKT pathway by ET-1 inhibits fibroblast apoptosis, and this inhibition is reversed by blockade of p38 MAPK or PI3K. TGF- β 1 has been shown to attenuate myofibroblast apoptosis through the p38 MAPK-dependent secretion of a soluble factor, which activates PI3K/AKT. In this study, we show that, although TGF- β 1 induces fibroblast synthesis and secretion of ET-1, TGF- β 1 activation of PI3K/AKT is not dependent on ET-1. We conclude that ET-1 and TGF- β 1 independently promote fibroblast resistance to apoptosis through signaling pathways involving p38 MAPK and PI3K/AKT. These findings suggest the potential for novel therapies targeting the convergence of prosurvival signaling pathways activated by these two profibrotic mediators.

Keywords: myofibroblast; fibrosis; Fas; p38 mitogen-activated protein kinase; mesenchymal cells

Endothelin (ET)-1 is a soluble fibrogenic peptide that has been implicated in the pathogenesis of pulmonary fibrosis. Secreted as a proenzyme, active ET-1 is generated through cleavage by ET-converting enzyme. The two $G_{\alpha_{q/11}}$ -coupled ET receptors (ET-A and ET-B) activate phospholipase (PL) C to initiate intracellular signaling events (1). Although ET-1 has been extensively studied in the pathobiology of pulmonary arterial hypertension, accumulating evidence supports a role for ET-1 in the pathogenesis of pulmonary fibrosis (2). Animal models show that increased ET-1 levels precede collagen deposition in the lungs after administration of intratracheal bleomycin, and that transgenic overexpression of ET-1 is sufficient to induce progressive pulmonary fibrosis (3, 4). Moreover, ET-1 levels are elevated in patients with pulmonary fibrosis from several

CLINICAL RELEVANCE

The primary finding of this study is that two fibrogenic growth factors, endothelin (ET)-1 and transforming growth factor (TGF)- β 1 independently activate antiapoptotic signaling mechanisms in human lung fibroblasts by p38 mitogen-activated protein kinase-dependent activation of phosphatidylinositol 3'-OH kinase/AKT. These results provide important insights into the mechanisms regulating fibroblast survival and apoptosis, with broad implications in the regulation of lung injury and repair processes. The convergence antiapoptotic signaling pathways mediated by ET-1 and TGF- β 1 may represent novel therapeutic targets for the treatment of pulmonary fibrosis.

underlying etiologies (5). *In vitro*, ET-1 has profibrotic effects on lung fibroblasts, promoting proliferation (6), differentiation (7), and extracellular matrix (ECM) synthesis (8, 9).

A key feature of pulmonary fibrosis is the accumulation of myofibroblasts, contractile mesenchymal cells with a phenotype that is intermediate between fibroblasts and smooth muscle cells, which synthesize, secrete, organize, and remodel the ECM (10). Myofibroblasts are critical effectors of wound-repair responses, and normal repair requires the ultimate clearance of myofibroblasts by apoptosis (10, 11). Insufficient myofibroblast apoptosis is associated with progressive ECM deposition and contraction, leading to tissue fibrosis (12).

We have previously shown that transforming growth factor (TGF)- β 1, a cytokine strongly associated with fibrosis in the lung and other organs/tissues, protects myofibroblasts from apoptosis by p38 mitogen-activated protein kinase (MAPK)-dependent secretion of a soluble factor that activates phosphatidylinositol 3'-OH kinase (PI3K)/AKT signaling (13). The PI3K/AKT signaling pathway is activated after ligation of receptor tyrosine kinases and seven transmembrane G protein-coupled receptors, or through integrin-associated adhesion-mediated signaling cascades (14). Activated AKT may support cell survival through a number of potential mechanisms, including regulation of BCL-2 family proteins, NF- κ B, caspase-9, and forkhead family transcription factors (14). TGF- β 1 has been shown to induce secretion of ET-1 by fibroblasts (15). This study was undertaken to investigate the role of ET-1 in the regulation of fibroblast fate and to determine if ET-1 mediates TGF- β 1 activation of PI3K/AKT and apoptosis resistance in fibroblasts. Portions of this work have been previously published in abstract form (16).

MATERIALS AND METHODS

Cells and Cell Culture

Normal primary human fetal lung fibroblasts (IMR-90; Institute for Medical Research, Camden, NJ) between passages 7 and 12 were

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cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin/streptomycin (Sigma, St. Louis, MO), fungizone (Invitrogen, Carlsbad, CA), and 5% FBS (Sigma). IMR-90 fibroblasts were incubated in 5% CO₂ at 37°C to 60–80% confluence, and growth arrested for 24 hours in DMEM with 0.01% FBS before treatment.

Primary fibroblast cultures were generated using methods previously described (17). Normal lung fibroblasts were cultured from histologically normal portions of lung from patients undergoing surgical resection of biopsy-proven non-small cell lung cancer. Idiopathic pulmonary fibrosis (IPF) fibroblasts were obtained via surgical lung biopsy or from lung explants from patients undergoing lung transplantation. For experiments using normal adult and IPF fibroblasts, three different normal and three different IPF fibroblast lines were used. All studies were approved by the Institutional Review Board at the University of Michigan.

Reagents

ET-1 (human, porcine) and cycloheximide were from Sigma. Porcine-derived TGF- β 1 and goat polyclonal antibodies to uncleaved and cleaved caspase-3 were from R&D Systems (Minneapolis, MN). SB203580, SP600125, U73122, Wortmannin, LY294002, and SB431542, along with antibodies to serine-473 phospho AKT, total AKT, phospho-p44/p42 extracellular signal-regulated kinase (ERK) MAPK, phospho p38 MAPK, phospho JNK MAPK, and the corresponding total MAPK antibodies were from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibodies to glyceraldehyde-3-phosphate dehydrogenase were from Abcam (Cambridge, MA). Secondary horseradish peroxidase-conjugated anti-mouse, anti-goat, and anti-rabbit antibodies were obtained from Pierce (Rockford, IL). Anti-Fas (activating) antibody clone CH11 and mouse monoclonal antibody to single-stranded DNA (ssDNA) were from Millipore (Billerica, MA).

Western Immunoblotting

Whole-cell lysates were collected in ice-cold RIPA buffer (1% nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M NaH₂PO₄, 2 mM EDTA, 0.5 mM NaF) containing 2 mM sodium orthovanadate and 1:100 dilution of protease inhibitor mixture III (Calbiochem, San Diego, CA). Protein estimation was done with the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Whole-cell lysates were reduced by mixing with a 1:5 vol/vol ratio of 6 $\times$ electrophoresis sample buffer (0.2 M EDTA, 40 mM dithiothreitol, 6% SDS, 0.06 mg/ml pyronin, pH 6.8) and boiling for 7 minutes. Equal amounts of protein were subjected to SDS-PAGE electrophoresis, as previously described (18).

Real-Time PCR

Total RNA was isolated using the TRIzol reagent, according to the manufacturer's instructions (Invitrogen). A total of 1.0 μ g RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen). The cDNA was then amplified by real-time quantitative TaqMan PCR using an ABI Prism 7,700 sequence detection system (Applied Biosystems, Foster City, CA); β -actin was used as an internal control. SYBR Green Master PCR mix (Applied Biosystems) was used to amplify ET-1, ET-A receptor (ET-A), and ET-B receptor (ET-B). Primers were as follows: (ET-1) forward, 5'-GCTCGTCCCTG ATGGATAAA-3', reverse, 5'-CTGTTGCCCTTTGTGGGAAGT-3'; (ETAR) forward, 5'-GCTTCCTGGTTACCACTCATCAA-3', reverse, 5'-TAGTCTGCTGTGGGCAATAGTTG-3'; (ETBR) forward, 5'-GCCAAGGACCCATCGAGAT-3', reverse, 5'-GAAGTGTGGA GTTCCCGATGAT-3'; (β -actin) forward, 5'-GCCAC GGCTG CTTCCA-3', reverse, 5'-GAACCGCTCATTGCCATTG-3'. Gene expression is shown as a fold increase in transcript expression in treated fibroblasts compared with untreated fibroblasts using the Δ Ct method, per manufacturer's instructions (Applied Biosystems).

Small Interfering RNA Transfections

RNA interference was accomplished by transfecting cells with ON-TARGET plus SMART pool small interfering RNA (siRNA) for

human ET-1 (EDN1;NM_001955), ET-A (EDNRA;NM_001957), and ET-B (EDNRB;NM_003991) from Dharmacon Inc. (Lafayette, CO). Transfections were performed using oligofectamine reagent (Invitrogen), according to the manufacturer's instructions. Briefly, for a 35-mm cell culture dish, 5 μ l of oligofectamine and 100 nm of siRNA were mixed for 20 minutes at room temperature. A 200- μ l aliquot of the oligofectamine/siRNA mixture was then combined with 800 μ l of Opti-Eagle's minimum essential medium for treatment of the cells. After 24 hours, the media were changed to DMEM with 5% FBS. Cells were incubated for 48 hours, and growth arrested in serum-free DMEM for 24 hours before treatment. Nontransfected cells and cells transfected with a nontargeting pooled siRNA (siControl) (Dharmacon Inc.) were used as controls.

ET-1 ELISA

Cell culture supernatants collected after treatments were used for quantitative determination of ET-1 levels by EIA kit from Assay Designs (Ann Arbor, MI), according to the manufacturer's instructions.

Assessments of Apoptosis

Apoptosis was assessed by Western immunoblotting for cleaved caspase-3 and ELISA for ssDNA, as previously described (19).

RESULTS

ET-1 Activates PI3K/AKT in Normal Lung Fibroblasts

Activation of PI3K/AKT by TGF- β 1 promotes myofibroblast resistance to apoptosis (13, 19, 20). To investigate the role of ET-1 in the regulation of fibroblast apoptosis, we first determined if ET-1 activated PI3K/AKT in normal human fetal lung fibroblasts. IMR-90 fibroblasts were treated with ET-1 at concentrations from 0 to 2,500 ng/ml for times ranging from 15 minutes to 24 hours, and AKT activity was assessed by Western immunoblotting with a specific antibody targeting S⁴⁷³ phosphorylation of AKT (Figure 1). A single treatment of ET-1 induced AKT phosphorylation within 1 hour. Maximal activation of AKT was seen within 3 hours, and AKT activation was maintained for 24 hours (Figure 1A). ET-1 induced AKT activation in normal lung fibroblasts at concentrations ranging from 25 to 2,500 ng/ml (10–1,000 nM), with optimal activation occurring at a concentration of 250 ng/ml (Figure 1B). As expected, AKT phosphorylation by ET-1 was blocked by inhibition of either PI3K or PLC (Figure 1B). Similar robust phosphorylation of AKT by 250 ng/ml ET-1 was seen in normal adult and IPF lung fibroblasts (Figure 2D).

ET-1 Activation of PI3K/AKT Is Mediated by p38 MAPK

MAPKs, including p38, ERK1/2, and JNK, have been shown to mediate the downstream signaling by ET-1 (21). To investigate the mechanism of PI3K/AKT activation by ET-1, IMR-90 fibroblasts were treated with ET-1 (250 ng/ml), and phosphorylation of these MAPKs was assessed at time points from 15 minutes to 6 hours (Figure 2A). Significant activation of p38 MAPK, ERK1/2, and JNK were observed within 15 to 30 minutes after treatment with ET-1. However, p38 MAPK phosphorylation plateaued after 15 to 30 minutes, whereas ERK1/2 and JNK phosphorylation increased gradually over the 6-hour time course studied (Figure 2A). To determine if these MAPKs had a role in ET-1 activation of PI3K/AKT, IMR-90 fibroblasts were treated for 3 hours with ET-1 in the presence/absence of specific inhibitors of these MAPK pathways, PI3K, or PLC, and AKT phosphorylation was determined. Inhibition of p38 MAPK, PI3K and PLC completely

Figure 1. Endothelin (ET)-1 activation of phosphatidylinositol 3'-OH kinase (PI3K)/AKT. Normal human fetal lung fibroblasts (IMR-90) were cultured on tissue culture plates to 80% confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS before growth arrest for 24 hours in DMEM with 0.01% FBS. (A) IMR-90 fibroblasts were treated with ET-1 (250 ng/ml) for the times indicated. (B) IMR-90 fibroblasts were treated for 3 hours with the concentration of ET-1

shown, or with 250 ng/ml ET-1 in the presence/absence of inhibitors of PI3K (Wortmannin, 50 nM) or phospholipase (PL) C (U73122, 5 μ M). At the conclusion of treatment, whole-cell lysates were collected and AKT phosphorylation was assessed by SDS-PAGE immunoelectrophoresis and Western immunoblotting. Membranes were stripped and probed for total AKT.

blocked ET-1 activation of AKT (Figure 2B). In contrast, inhibition of ERK1/2 or JNK did not inhibit, and appeared to increase, ET-1 phosphorylation of AKT. In separate experiments, blockade of the type-1 TGF- β receptor (ALK5) had no significant effect on AKT activation by ET-1 (Figure 2C). Similarly, inhibition of p38 MAPK blocked ET-1 activation of AKT in primary adult normal and IPF lung fibroblasts (Figure 2D).

Figure 2. AKT phosphorylation by ET-1 requires p38 mitogen-activated protein kinase (MAPK). (A) Quiescent IMR-90 fibroblasts were treated with ET-1 (250 ng/ml) for the time periods indicated. Whole-cell lysates were assessed by SDS-PAGE immunoelectrophoresis and Western immunoblotting for: phospho p38 MAPK, phospho extracellular signal-regulated kinase (ERK) 1/2 MAPK, and phospho JNK MAPK. Membranes were stripped and probed for the corresponding total MAPK. (B) IMR-90 fibroblasts were treated with ET-1 (250 ng/ml) for 3 hours in the presence/absence of inhibitors of p38 MAPK (SB203580, 6 μ M), JNK MAPK (SP600125, 100 nM), ERK1/2 (PD98059, 20 μ M), PI3K (Wortmannin 50 nM), or PLC (U73122, 5 μ M). Whole-cell lysates were assessed for AKT phosphorylation by Western immunoblotting, and the membrane was stripped and probed for total AKT. (C) IMR-90 fibroblasts were treated with ET-1 (250 ng/ml) in the presence/absence of SB431542 (1 μ M), an inhibitor of the type-1 TGF- β receptor (ALK5). Cell lysates obtained at 3 hours were assessed for AKT phosphorylation by Western immunoblotting, and the membrane was stripped and probed for total AKT. (D) Primary normal adult lung fibroblasts (left

panel) and idiopathic pulmonary fibrosis (IPF) fibroblasts (right panel) were treated with ET-1 (250 ng/ml) for 3 hours in the presence/absence of inhibitors of p38 MAPK (SB203580, 6 μ M), PI3K (Wortmannin 50 nM), or PLC (U73122, 5 μ M). AKT phosphorylation was assessed by Western immunoblotting, and membranes were stripped and probed for total AKT. Data represent three normal and three IPF fibroblast cell lines.

ET-1 Attenuates Fibroblast Apoptosis

We next sought to determine if ET-1 activation of PI3K/AKT would confer an apoptosis-resistant phenotype to lung fibroblasts. Previous reports have shown fibroblast resistance to extrinsic apoptosis triggered by ligation of the Fas/CD95 receptor (22–24). However, fibroblast susceptibility to Fas-mediated apoptosis can be restored by sensitization with TNF- α , or by the addition of cycloheximide, an inhibitor of protein translation (22, 24). To determine the susceptibility of IMR-90 lung fibroblasts to apoptosis induced by Fas-activation, IMR-90 fibroblasts were treated with CH11, a Fas-activating antibody (FasL), in the presence/absence of cycloheximide, and apoptosis was measured by ELISA for ssDNA (Figure 3A) and by Western immunoblotting for cleaved caspase 3 (Figure 3B). Consistent with the previous reports, we found that IMR-90 fibroblasts were resistant to Fas-mediated apoptosis, and that susceptibility to apoptosis was restored in the presence of cycloheximide. As prior studies have reported variable susceptibility of normal and IPF lung fibroblasts after stimulation with FasL (22, 23), we additionally assessed apoptosis in normal adult and IPF lung fibroblasts (Figure 3C). Similar to the results in IMR-90 fibroblasts, apoptosis in the primary adult lung and IPF fibroblasts was only induced by the combination of Fas-activating ligand and cycloheximide.

To determine if ET-1 modified apoptosis susceptibility, and to assess the role of PI3K/AKT by ET-1 in fibroblast survival, cells were treated with the combination of FasL and cycloheximide in the presence/absence of ET-1 with/without inhibitors of PLC, p38 MAPK, or PI3K (Figure 4). IMR-90 fibroblasts (Figures 4A–4C), normal primary adult lung fibroblasts (Figure 4D, *left panel*), and IPF fibroblasts (Figure 4D, *right panel*) all demonstrated significantly decreased apoptosis after treatment with ET-1. In each fibroblast cell line, the antiapoptotic effects conferred by ET-1 were lost after blockade of PLC, p38 MAPK, or PI3K. Interestingly, inhibition of PI3K enhanced the apoptotic response to Fas

and cycloheximide in IMR-90 fibroblasts (Figure 4A), but not in adult (normal or IPF) fibroblasts (Figure 4D). These findings support a novel prosurvival mechanism of ET-1 mediated by p38 MAPK and PI3K/AKT in lung fibroblasts.

TGF- β 1 Activation of PI3K/AKT Protects Fibroblasts from Apoptosis Induced by Fas Activation

We have shown that p38 MAPK is necessary for TGF- β 1 activation of PI3K/AKT in IMR-90 fibroblasts and alveolar mesenchymal cells from the bronchoalveolar lavage fluid of patients with acute respiratory distress syndrome (ARDS) (13). To determine if a similar signaling pathway is activated in normal adult lung fibroblasts and IPF fibroblasts, these primary cells were treated with TGF- β 1 in the presence or absence of an inhibitor of p38 MAPK or an ALK5 inhibitor, and AKT phosphorylation was assessed after 16 hours. Consistent with IMR-90 and ARDS fibroblasts, inhibition of p38 MAPK blocked TGF- β 1 activation of PI3K/AKT (Figure 5A). We next showed that TGF- β 1 activation of AKT-attenuated apoptosis induced by FasL and cycloheximide in IMR-90 fibroblasts (Figure 5B). Consistent with the previous experiment (Figure 4A), apoptosis of IMR-90 fibroblasts was enhanced by inhibition of PI3K along with the combination of Fas and cycloheximide compared with the combination of Fas and cycloheximide alone. As we found that TGF- β 1 and ET-1 confer similar degrees of protection from apoptosis via activation of PI3K/AKT, we next sought to determine the relationship between fibrogenic TGF- β 1 and ET-1 in the regulation of fibroblast apoptosis.

TGF- β 1 Induces ET-1 Expression in Lung Fibroblasts

ET-1 is a soluble factor that activates PI3K/AKT, and protects fibroblasts from apoptosis. To determine if autocrine activation of PI3K/AKT by TGF- β 1 is mediated by TGF- β 1-induced secretion of ET-1, we examined the regulation of ET-1 by TGF- β 1 in lung fibroblasts. RNA and cell culture supernatants were collected from IMR-90 fibroblasts between 0 and 24 hours

Figure 3. Fibroblast apoptosis requires combined treatment with FasL and cycloheximide. (A) IMR-90 fibroblasts were cultured in a 96-well plate to 80% confluence, and growth arrested for 24 hours before treatment with/without Fas-activating antibody (FasL; CH11, 250 ng/ml), cycloheximide (500 ng/ml), or the combination of FasL and cycloheximide for 16 hours. Apoptosis was assessed by ELISA for single-stranded DNA (ssDNA), and is normalized to the level of untreated controls. $P < 0.001$ for the combination of FasL plus cycloheximide compared with untreated controls. (B) IMR-90 fibroblasts in 35-mm cell culture dishes were similarly treated, and apoptosis was assessed by Western immunoblotting of whole-cell lysates for cleaved caspase-3. The membranes were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The data shown are representative of three separate experiments. (C) Primary normal adult fibroblasts (*left panel*) and IPF fibroblasts (*right panel*) were similarly treated, and apoptosis assessed by Western immunoblotting for cleaved caspase-3. Membranes were stripped and probed for GAPDH. The data shown are representative of experiments done with three normal and three IPF fibroblast cell lines.

Figure 4. ET-1-mediated protection from apoptosis is dependent on p38 MAPK and PI3K/AKT. (A–C) IMR-90 fibroblasts in a 96-well plate were treated with the combination of FasL (250 ng/ml) and cycloheximide (500 ng/ml) in the presence/absence of ET-1 (250 ng/ml) with/without inhibitors of PLC (U73122, 5 mM), p38 MAPK (SB203580, 6 μ M), or PI3K (Wortmannin, 50 nM) for 16 hours. Apoptosis was assessed by (A) ELISA for ssDNA (four replicates per condition and three separate experiments) and (B) Western immunoblotting for cleaved caspase-3. The membrane in (B) was stripped and probed for GAPDH. (C) Densitometric analysis of cleaved caspase-3/GAPDH ratios from three independent experiments. (D) Similarly treated primary normal lung fibroblasts (*left panel*) and IPF fibroblasts (*right panel*) were assessed for apoptosis by ELISA for ssDNA ($n = 3$ normal lung fibroblast lines and 3 IPF fibroblast lines).

after treatment with TGF- β 1. Quantitative real-time PCR showed that TGF- β 1 strongly up-regulates ET-1 transcription, with maximal mRNA expression noted between 6 and 16 hours (Figure 6A). Basal levels of active ET-1 in cell culture supernatants were undetectable, and stimulation with TGF- β 1 led to a small, but significant, increase in active ET-1 (Figure 6B).

Similar induction of ET-1 by TGF- β 1 was found in normal adult and IPF lung fibroblasts (Figure 7C).

TGF- β 1 Activation of PI3K/AKT Is Not Mediated by ET-1

TGF- β 1 activation of PI3K/AKT requires the p38 MAPK-dependent secretion of a soluble factor (13). Thus, if ET-1

Figure 5. TGF- β 1 activation of PI3K/AKT is dependent on p38 MAPK and protects fibroblasts from apoptosis induced by FasL and cycloheximide. (A) Primary normal adult lung fibroblasts (left panel) and IPF fibroblasts (right panel) were treated with TGF- β 1 (2 ng/ml) in the presence/absence of inhibitors of p38 MAPK (SB203580, 6 μ M) or ALK5 (SB431542, 1 μ M) for 16 hours. AKT phosphorylation was assessed by Western immunoblotting. Membranes were stripped and probed for total AKT. Data shown represent three normal and three IPF fibroblast cell lines. (B) IMR-90 fibroblasts were treated with the combination of FasL (250 ng/ml) and cycloheximide (500 ng/ml) with/without TGF- β 1 (2 ng/ml) alone or with an inhibitor of PI3K (LY294002, 10 μ M) for 16 hours. Apoptosis was assessed by ELISA for ssDNA, and is normalized to the untreated control.

mediates TGF- β 1 activation of PI3K/AKT, it should be regulated by TGF- β 1 in a p38 MAPK-dependent manner. To define the role of ET-1 in TGF- β 1 activation of PI3K/AKT, we treated fibroblasts with TGF- β 1 in the presence or absence of a p38 MAPK inhibitor (Figure 7). Surprisingly, inhibition of p38 MAPK had no significant effect on TGF- β 1 induction of ET-1 transcription at 6 hours (Figure 7A), or on the secretion of

active ET-1 at 16 hours (Figure 7B). Moreover, inhibition of p38 MAPK did not impact TGF- β 1 induction of ET-1 in normal adult or IPF-lung fibroblasts (Figure 7C). As inhibition of p38 MAPK failed to block TGF- β 1-induced synthesis or secretion of ET-1, these data suggest that ET-1 was not the soluble factor directly mediating TGF- β 1 activation of PI3K/AKT.

To confirm that TGF- β 1 activation of PI3K/AKT was independent of ET-1, we employed siRNA to knock down ET-1 or its receptors, ET-A and ET-B, and examined the impact on TGF- β 1 activation of AKT. Knockdown of ET-1 or its receptors was confirmed by real-time PCR 72 hours after siRNA transfection of IMR-90 fibroblasts compared with the expression of cells transfected with a nontargeting (control) siRNA (Figure 8A). Transfected fibroblasts were then treated with/without TGF- β 1 for 16 hours, and AKT activation was assessed. As seen in Figure 8B, AKT phosphorylation by TGF- β 1 was not affected by siRNA knockdown of ET-1, ET-A, or ET-B, confirming that neither ET-1 nor its receptors are required for TGF- β 1 activation of PI3K/AKT.

DISCUSSION

TGF- β 1 and ET-1 have been implicated in the pathogenesis of pulmonary fibrosis. Both cytokines have been shown to modulate pulmonary fibrosis in murine models, and both have been shown to be highly expressed in the lungs of patients with pulmonary fibrosis (4, 25–29). Moreover, both of these mediators induce fibroblast differentiation, ECM synthesis, and collagen gel contraction (7, 10). TGF- β 1 has been shown to promote myofibroblast resistance to apoptosis (13, 19, 20, 30, 31), but few studies have addressed the effects of ET-1 on this profibrotic fibroblast phenotype. This study was undertaken to examine the effects of ET-1 on fibroblast survival signaling and apoptosis.

First, we examined the mechanism of ET-1 activation of PI3K/AKT in normal and IPF lung fibroblasts. Next, we investigated the role of PI3K/AKT activation by ET-1 in the

Figure 6. ET-1 expression is increased by TGF- β 1 treatment. Quiescent IMR-90 fibroblasts were treated with TGF- β 1 (2 ng/ml) for the times indicated. (A) RNA was isolated and ET-1 expression was assessed by quantitative real-time PCR ($n = 3$). (B) Active ET-1 in the cell culture supernatants was assessed by ELISA ($n = 3$). * $P < 0.001$ compared with untreated controls.

Figure 7. ET-1 induction by TGF-β1 is not dependent on p38 MAPK. (A and B) IMR-90 fibroblasts were treated with TGF-β1 (2 ng/ml) in the presence/absence of a p38 MAPK inhibitor (SB203580, 6 μM). (A) RNA was collected at 6 hours, and ET-1 expression was assessed by quantitative real-time PCR. (B) Cell culture supernatants were collected at 16 hours and ET-1 levels assessed by ELISA ($n = 3$) for (A) and (B). * $P < 0.001$ compared with untreated controls. (C) Primary normal adult fibroblasts (open bars) and IPF fibroblasts (closed bars) were treated with TGF-β1 (2 ng/ml) with/without a p38 MAPK inhibitor (SB203580, 6 μM) for 16 hours and soluble ET-1 was assessed by ELISA. $n = 3$ normal and 3 IPF fibroblast lines. * $P < 0.001$ compared with untreated controls).

regulation of fibroblast survival/apoptosis. Finally, we sought to determine if ET-1 functioned as a downstream mediator of TGF-β1 in the regulation of fibroblast survival, or if ET-1 functioned in a parallel, independent manner. Our primary findings are that ET-1 robustly activates PI3K/AKT in a p38 MAPK-dependent manner, and that ET-1 activation of PI3K/AKT confers apoptosis resistance to fibroblasts. In addition, although TGF-β1 induces ET-1 expression in fibroblasts, ET-1 is not required for TGF-β1 activation of PI3K/AKT. Collectively, these studies establish that TGF-β1 and ET-1 independently promote fibroblast survival through p38 MAPK-dependent activation of PI3K/AKT.

Prevailing hypotheses pose that pulmonary fibrosis results from dysfunctional repair after lung injury from known causes, as in scleroderma and ARDS, or unknown causes, as in IPF (32). Regardless of etiology, pulmonary fibrosis is characterized by

myofibroblast accumulation and excessive deposition of ECM. After injury, recruited fibroblasts differentiate into myofibroblasts, which synthesize, secrete, organize, and contract the ECM (10, 33). The fate of recruited fibroblasts may be a critical determinant of the outcome of wound repair, as normal resolution requires the apoptotic clearance of myofibroblasts, whereas fibrosis is associated with insufficient myofibroblast apoptosis (11, 12, 33, 34). Although the current study was not designed to evaluate differences in the apoptosis susceptibility of normal and fibrotic fibroblasts, several studies have found that fibroblasts isolated from the patients with pulmonary fibrosis of different etiologies have increased resistance to apoptosis compared with normal lung fibroblasts (20, 22, 23, 35).

The physiologic stimuli of myofibroblast apoptosis during the resolution phase of wound repair have not been clearly defined. Consistent with several prior studies, we found that lung fibroblasts demonstrate resistance to extrinsic apoptosis induced by ligation of the membrane-bound death receptor, Fas/CD95 (22, 24, 35). In contrast, one study demonstrated fibroblast apoptosis susceptibility to Fas activation, but used a significantly higher concentration of Fas-activating ligand (23). Our study demonstrates fibroblast resistance to apoptosis induced by Fas, which is overcome by cotreatment with cycloheximide, an inhibitor of protein translation. The mechanism of fibroblast resistance to Fas-mediated apoptosis remains poorly defined, and several mechanisms have been proposed. Tanaka and colleagues (24) found that resistance to Fas-mediated apoptosis was due to increased constitutive expression of inhibitors of apoptosis, and that cycloheximide blocked that constitutive expression, allowing apoptosis to proceed. Frankel and colleagues (22) showed that TNF-α facilitates recruitment of the Fas-associated death domain to the cytoplasmic domain of Fas, thereby promoting apoptosis.

The regulation of fibroblast survival and apoptosis in lung homeostasis and disease remains poorly understood, but evidence supports a significant role for PI3K/AKT signaling. TGF-β1 activation of PI3K/AKT, and subsequent myofibroblast resistance to apoptosis, is mediated by the p38 MAPK-dependent, SMAD-independent secretion of a putative soluble factor (13, 19). Focal adhesion kinase, a nonreceptor tyrosine kinase that is activated by TGF-β1, has also been shown to induce apoptosis resistance in fibroblasts through activation of PI3K/AKT (18, 36). Murine models show that phosphorylated AKT is strongly expressed in areas of pulmonary fibrosis after intratracheal administration of bleomycin, and that blockade of PI3K/AKT signaling attenuates pulmonary fibrosis induced by intratracheal bleomycin or TGF-β1 overexpression (37, 38). Finally, increased AKT activation in primary lung mesenchymal cells is associated with resistance to apoptosis and a clinical course of persistent ARDS (20).

In this study, we show that ET-1 inhibits fibroblast apoptosis through activation of PI3K/AKT, suggesting a novel fibrogenic mechanism of ET-1. ET-1 has been shown to inhibit cancer cell apoptosis by activation of PI3K/AKT (39). However, no prior studies have examined the role of ET-1 activation of PI3K/AKT in lung fibroblast survival. One study reported that PI3K/AKT regulates ET-1-induced fibroblast differentiation and contraction (7). Another study recently showed that fibroblasts lacking Thy-1 have decreased apoptosis in contractile collagen gels after treatment with ET-1 when compared with ET-1-treated fibroblasts expressing Thy-1 (40). Our studies show that ET-1 protects normal fetal, normal adult, and IPF lung fibroblasts from apoptosis through activation of PI3K/AKT. Interestingly, the current study suggests that blockade of PI3K enhances Fas-mediated apoptosis in fetal, but not adult, lung fibroblasts (Figures 4A, 4D, and 5B). The mechanism of enhanced apoptosis is unclear, but these data suggest that constitutive

Figure 8. TGF- β 1 activation of PI3K/AKT is independent of ET-1. IMR-90 fibroblasts were transfected with pooled small interfering RNA (siRNA) targeting ET-1, the ET-A receptor, the ET-B receptor, or with a nontargeting (NT) siRNA. (A) At 72 hours after transfection, RNA was isolated from the transfected cells for quantitative real-time PCR for ET-1, ET-A, or ET-B to demonstrate that there was significant knockdown of the targeted mRNA. (B) Similarly transfected IMR-90 fibroblasts, or non-transfected fibroblasts, were treated with/without TGF- β 1 (2 ng/ml) for 16 hours, and AKT phosphorylation was assessed by Western immunoblotting. Membranes were stripped and probed for total AKT. Results are representative of three separate experiments.

expression of PI3K-mediated prosurvival signals contributes to apoptosis resistance in fetal lung fibroblasts.

In concordance with previous reports, we found that ET-1 activates p38, ERK1/2, and JNK MAPKs (21, 41). Early activation of p38 MAPK is required for ET-1 activation of PI3K/AKT, whereas blockade of ERK1/2 or JNK fails to inhibit ET-1 activation of PI3K/AKT. Consistently, blockade of p38 MAPK reversed the antiapoptotic effects of ET-1. This study is the first to demonstrate that p38 MAPK is required for ET-1 activation of PI3K/AKT and acquisition of apoptosis resistance in mesenchymal cells. Collectively, these findings support a novel mechanism by which ET-1 contributes to the pathobiology of pulmonary fibrosis.

TGF- β 1 is associated with fibrosis of most organ systems studied, including the lung (42). Among its fibrogenic actions, TGF- β 1 induces fibroblast resistance to apoptosis through p38 MAPK-dependent secretion of a soluble factor that activates PI3K/AKT (13). In normal and scleroderma lung fibroblasts, autocrine secretion of ET-1 by TGF- β 1 has been found to promote fibroblast differentiation, collagen gel contraction, and ECM synthesis (15, 43). In the current study, we show that ET-1 confers protection from apoptosis in fibroblasts through activation of PI3K/AKT, and we questioned if autocrine induction of ET-1 mediates TGF- β 1 activation of PI3K/AKT. Contrary to our original hypothesis, our studies show that TGF- β 1 activation of PI3K/AKT is not mediated by ET-1. Although we found that TGF- β 1 does induce expression of ET-1 at the gene and protein levels, the absolute increase in soluble, active ET-1 is several orders of magnitude lower than the concentrations required for *in vitro* activation of AKT in fibroblasts. The observed concentrations of active ET-1 are consistent with those reported in scleroderma fibroblasts exposed to TGF- β (7, 15). Similar concentrations of ET-1 were found in the epithelial lining fluid of patients with ARDS and bronchoalveolar lavage fluid of patients with interstitial lung disease (44–46). Although the concentration of ET-1 secreted by mesenchymal cells stimulated with TGF- β 1 *in vitro* approximates these levels reported *in vivo*, the effective concentration of ET-1 within fibrotic tissue remains unclear, and may be markedly higher within the pericellular microenvironment.

Our studies indicate that TGF- β 1 induction of ET-1 is not inhibited by blockade of p38 MAPK, and that siRNA-mediated knockdown of ET-1 or its receptors (ET-A or ET-B) has no significant effect on TGF- β 1 activation of PI3K/AKT. Thus, although ET-1 and TGF- β 1 use the same signaling intermediates—p38 MAPK and PI3K/AKT—they independently promote fibroblast survival. Additionally, although the biologic

source of ET-1 in patients with IPF has not been defined, our studies suggest that ET-1 secreted by epithelial, endothelial, or inflammatory cells may activate fibroblast survival signaling in a paracrine manner (27, 47).

Effective pharmacologic therapies for pulmonary fibrosis are lacking, and current concepts favor therapies targeting fibrogenic mechanisms, including inhibition of TGF- β 1 and ET-1 (32, 48). Studies with ET receptor antagonists in murine models of pulmonary fibrosis have generated conflicting results (49, 50). In one study, a dual ET receptor antagonist reduced the accumulation of connective tissue in mouse lungs 28 days after intratracheal bleomycin (50). A different dual ET receptor antagonist, however, had no significant effect on lung collagen content in rats at 14 or 21 days after bleomycin administration (49). Recently, a clinical trial of a dual ET receptor antagonist in IPF failed to meet the primary efficacy endpoints; however, patients in the treatment group demonstrated a trend toward delayed death and disease progression (51). Further clinical trials of ET receptor antagonists for IPF are ongoing (clinicaltrials.gov: NCT00768300 and NCT00391443). Additionally, ongoing clinical trials are investigating anti-TGF- β 1 monoclonal antibodies in IPF (clinicaltrials.gov: NCT00125385). The current study shows that these two fibrogenic mediators—ET-1 and TGF- β 1—use the same intracellular signaling intermediates to independently induce an apoptosis-resistant fibroblast phenotype. These findings support the hypothesis that the acquisition of a profibrotic mesenchymal cell phenotype, in the context of pulmonary fibrosis, may be the result of several mediators acting alone or in combination (52). It remains to be seen if individuals with pulmonary fibrosis have disease states dominated by a single mediator (i.e., TGF- β 1 or ET-1), or if several mediators function in an additive or synergistic manner to induce fibrosis. Our data suggest that therapy targeting the convergence of downstream signaling pathways common to fibrogenic mediators, rather than targeting a single mediator, may provide improved clinical efficacy for the treatment of pulmonary fibrosis.

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