Idiopathic pulmonary fibrosis (IPF; a progressive lung disease) is characterized by parenchymal remodeling with enlarged air spaces called honeycomb cysts and palisades of fibroblasts called fibroblast foci. In IPF, lung epithelial cells covering honeycomb cysts and fibroblast foci aberrantly express the active conformation of the potent fibrogenic cytokine transforming growth factor-β1 (TGF-β1). Using explanted rat lung slices, we transfected alveolar epithelial cells with the retrovirus pMX containing a site-directed mutation in which Cys²²³ and Cys²²⁵ were substituted with serines, resulting in release of biologically active TGF-β1 and fibroblast proliferation and remodeling that resembled IPF. Fibroblasts obtained from transplanted explants and in culture for 6 weeks incorporated [³H]thymidine more [³H]thymidine compared with control fibroblasts without transfection or fibroblasts obtained from transplanted explants cultured with antibody to fibroblast growth factor-2 (FGF-2). Primary lung fibroblasts obtained from normal rat lungs cultured with TGF-β1 expressed increased levels of phosphorylated p38 MAPK and JNK, but not ERK1/2. The presence of TGF-β1 caused an immediate release of extracellular FGF-2 from primary pulmonary fibroblasts; and in the presence of anti-FGF-2 antibody, phosphorylated p38 MAPK and JNK were abrogated. TGF-β1 inhibits cell proliferation by suppression of c-Myc and induction of p15INK4b, p21CIP1, or p27KIP. Fibroblasts cultured with TGF-β1 showed no regulation of c-Myc or induction of p15INK4b, p21CIP1, or p27KIP. These findings suggest that pulmonary fibroblasts may not respond to the anti-proliferative effects of TGF-β1, but proliferate in response to TGF-β1 indirectly by the release of FGF-2, which induces phosphorylation of p38 MAPK and JNK.

The most common pulmonary fibrotic disorder is idiopathic pulmonary fibrosis (IPF), a progressive and lethal disease of unknown etiology and uncertain pathogenesis (1). The incidence of IPF is ~15–30 cases/100,000 persons/year (1–3). The histological findings in IPF are called usual interstitial pneumonia and are characterized by temporal heterogeneity (1–4) in which the normal appearing lung is seen adjacent to interstitial fibrosis, honeycomb cysts that are distorted, and enlarged airspaces and fibroblast foci (1, 4). Fibroblast foci are small aggregates of actively proliferating fibroblasts and myofibroblasts that are surrounded by the extracellular matrix (ECM) (1, 4). In IPF, fibroblast foci are widely dispersed, and their numbers correlate with worsening lung function, progression of disease, and poor prognosis (5, 6).

Using lung sections from patients with IPF, we previously demonstrated by immunohistochemistry that the biologically active conformation of transforming growth factor-β1 (TGF-β1), a potent fibrogenic cytokine, is expressed in epithelial cells lining honeycomb cysts in areas of advanced disease and remodeling (7–9). In regions of the lung where fibroblast foci are present, the biologically active conformation of TGF-β1 is observed in the hyperplastic pneumocytes overlying the fibroblast foci (7, 8). Alveolar epithelial cells (AECs) in lung sections with no evidence of inflammation or fibrosis do not express the active conformation of TGF-β1.

Based on our observations in IPF lungs demonstrating that AECs that line areas of fibrosis and fibroblast foci aberrantly express biologically active TGF-β1, we designed an in vitro model to determine the role of AEC-derived TGF-β1 in interstitial pulmonary fibrosis and parenchymal remodeling (10). In this model, we used slices of normal rat lungs, which were free of inflammatory cells. The AECs in the explanted lung slices were successfully transfected with the retrovirus pMX carrying an insert of TGF-β1 designated as L-s223,225-TGF-β1 (10). The L-s223,225-TGF-β1 cDNA contains a mutation in the TGF-β1 gene in which Cys²²³ and Cys²²⁵ have been substituted with serines (10). This mutation results in constitutive release of biologically active TGF-β1 (23). Retroviruses such as pMX enter only actively proliferating cells (10). To selectively transfect the AECs of the explant, the explants were treated with keratinocyte growth factor (KGF), a potent AEC mitogen (10). In situ hybridization using a digoxigenin-labeled probe of the puromycin resistance gene contained in the pMX retrovirus confirmed that, in the presence of KGF, only AECs of the explant were transfected with the vector (10). In lung explants in which the AECs were successfully transfected with the L-s223,225-TGF-β1 gene, there was release of active TGF-β1 and extensive fibrosis with lesions that resembled fibroblast foci and honeycomb cysts (10).

The main sources of connective tissue proteins are fibroblasts and collagenous matrix, and their functions are mediated by growth factors such as TGF-β1. TGF-β1 signaling is mediated by type II TGF-βR (TβR II) that mediates the activation of type I TGF-βR (TβR I). TβR I phosphorylates Smad proteins, which then translocate to the nucleus and regulate the expression of target genes (11).

**References**

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2. Primary lung fibroblasts obtained from normal rat lungs cultured with TGF-β1 showed no regulation of c-Myc or induction of p15INK4b, p21CIP1, or p27KIP.
3. Fibroblasts cultured with TGF-β1 showed no regulation of c-Myc or induction of p15INK4b, p21CIP1, or p27KIP.
4. These findings suggest that pulmonary fibroblasts may not respond to the anti-proliferative effects of TGF-β1, but proliferate in response to TGF-β1 indirectly by the release of FGF-2, which induces phosphorylation of p38 MAPK and JNK.
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6. AECs, alveolar epithelial cells; KGF, keratinocyte growth factor; FGF-2, fibroblast growth factor-2; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; CKIIs, cyclin-dependent kinase inhibitors; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; PDGF, platelet-derived growth factor; IGF-1, insulin-like growth factor-1; SAKP, stress-activated protein kinase; ERK1/2, extracellular signal-regulated kinase-1/2; TCD50, tissue culture infectious dose; TβR, transforming growth factor-β1 receptor; TBS, Tris-buffered saline.
myofibroblasts (11), which are increased in pMX-L-s223,225-TGF-β1-transfected explants (10). These findings suggest that the release of active TGF-β1 by AECs in this model directly or indirectly regulates the proliferation of interstitial pulmonary fibroblasts. Because the appearance of fibroblasts (especially in fibroblast foci) is associated with the pathogenesis of IPF and correlates with progression of fibrosis in IPF (5, 6), the current model provides an opportunity to determine the mechanism by which interstitial fibroblasts increase in number in response to AEC-derived TGF-β1 (7–10).

In this study, we demonstrate that fibroblasts obtained from lung tissue of explants with successful transfection of AECs with pMX-L-s223,225-TGF-β1 compared with control fibroblasts proliferated actively. However, when fibroblasts were obtained from transfected explants that were cultured with antibody to TGF-β1 or fibroblast growth factor-2 (FGF-2), DNA synthesis was decreased. Primary lung fibroblasts obtained from normal rat lungs cultured with TGF-β1 caused an almost immediate release of extracellular FGF-2 and expression of increased quantities of phosphorylated p38 MAPK and JNK; but in the presence of antibody to FGF-2, phosphorylation of p38 MAPK and JNK was totally abrogated. TGF-β1 inhibited proliferation of epithelial and hematopoietic cells by suppression of c-Myc and induction of cyclin-dependent kinase inhibitors (CKIs) p15INK4a, p21CIP1, and p27KIP. Fibroblasts cultured with TGF-β1 showed no regulation of c-Myc, p15INK4a, p21CIP1, or p27KIP. These findings suggest that TGF-β1 regulates the proliferation of pulmonary fibroblasts indirectly by the release of FGF-2, which, by induction of phosphorylation of p38 MAPK and JNK, leads to fibroblast proliferation. Furthermore, fibroblasts do not respond to the anti-proliferative effects of TGF-β1 by suppression of c-Myc and induction of CKIs p15INK4a, p21CIP1, and p27KIP.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum were purchased from Invitrogen (Burlington, Ontario, Canada). Agarose, hydrocortisone, retinol acetate, insulin-transferrin-sodium selenium, and fetuin were purchased from Sigma. Recombinant KGF/FGF-7, the TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit, porcine TGF-β1, anti-FGF-2 antibody, anti-human platelet-derived growth factor (PDGF) antibody, anti-human insulin-like growth factor-1 (IGF-1) antibody, and neutralizing anti-TGF-β1 antibody were purchased from R&D Systems (Minneapolis, MN). Antibodies to detect total p38 MAPK, SAPK/JNK, and ERK1/2 were purchased from Stressgen (Victoria, British Columbia, Canada), New England Biolabs Inc. (Pickering, Ontario), and Upstate Cell Signaling Solutions (Charlottesville, VA), respectively. Anti-phospho-Thr183/Tyr185 SAPK/JNK, anti-phospho-Thr180/Tyr182 p38 MAPK, anti-phospho-Thr202/Tyr204 p44/42 MAPK, and anti-phospho-ERK1/2 (E10) antibodies were purchased from Cell Signaling (Beverly, MA). Anti-c-Myc antibody 9E10 and anti-p15INK4a antibody K-18 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-p21CIP1 and anti-p27KIP antibodies were purchased from Pharmingen (Mississauga, Ontario). Anti-vimentin and biotinylated, rat-absorbed rabbit anti-mouse antibodies were purchased from Dako Corp. (Carpinteria, CA). The L2, C3H/10T1/2, and NIH/3T3 cell lines were obtained from American Type Culture Collection (Manassas, VA).

Preparation of the Retroviral Vector—pPK9A (a gift from Dr. Lalage Wakefield, Laboratory of Chemoprevention, National Institutes of Health, Bethesda, MD) is a plasmid that contains the entire length of the cDNA of TGF-β1 with Cys223 and Cys325 substituted with serines (12). The substitution of cysteines with serines at these positions results in secretion of TGF-β1 in its biologically active form (10, 12), and this cDNA was named L-s223,225-TGF-β1 (10). Using BglII digestion, the 1.2-kb TGF-β1 was isolated and purified with a DNA gel extraction kit (Qiagen Inc., Mississauga) (10). L-s223,225-TGF-β1 was subcloned into the retroviral vector pMX (a gift from Dr. Alice Mui, Department of Surgery, University of British Columbia) (10). The pMX retrovirus with no TGF-β1 cDNA was designated as pMX, whereas the retrovirus containing the TGF-β1 gene was designated as pMX-L-s223,225-TGF-β1 (10). The retroviruses were produced using the packaging cell line Plat-E, followed by quantitation based on the number of infected NIH/3T3 cells (10).

Preparation of Normal Rat Lung Slices—Female Sprague-Dawley rats that were free of respiratory disease and that weighed 200–250 g were purchased from the University of British Columbia Vivarium. Intrap eritoneal administration of 0.4 ml of ketamine (Biomedia-MTC, Cambridge, Ontario) and 0.2 ml of Rompun (Bayer, Etobicoke, Ontario) was used for euthanization. The trachea was exposed, and an 18-gauge catheter was inserted through the cartilaginous rings. After exposure of the thoracic and abdominal cavities, the inferior vena cava and abdominal aorta were severed. The peripheral blood was removed with 10 ml of normal saline injected into the right ventricle until the lungs turned white. The trachea and lungs were taken out. To remove alveolar cells, the lungs were lavaged with 50–60 ml of warm normal saline through the trachea and then infused with 5 ml of 0.4% agarose/DMEM (2× solution of 1:1 serum-free DMEM and 0.8% agarose (Invitrogen)) at 40 °C. The medium was supplemented with hydrocortisone (0.2 μg/ml), retinol acetate (0.2 μg/ml), and 0.02% insulin-transferrin-sodium selenium. A thread was used to tie the tracheas, and lungs were placed on culture dishes on ice overnight to further solidify the lungs. The lungs were sliced manually at 1–2-mm thickness from each lobe with a sterilized scalpel (10). In some instances, alveolar macrophages were isolated from the lavage fluid, cultured as described previously (7), and stained with vimentin.

Culture of Rat Lung Slices—1.5 ml of warm 0.4% agarose/DMEM was added to each 6-well plate. After the agarose/DMEM solidified, four to six lung slices were placed on top of the agarose, and 1.5 ml of serum-free DMEM supplemented with hydrocortisone (0.2 μg/ml), retinol acetate (0.2 μg/ml), and 0.02% insulin-transferrin-sodium selenium was added. The lung slices were incubated at 37 °C under 5% CO2. The medium was changed twice each week, and lung slices were turned every other day and collected after culture. Treatment of the lung slices consisted of medium, KGF (25 ng/ml), pMX (106 TCID50), or pMX-L-s223,225-TGF-β1 (106 TCID50) + KGF (25 ng/ml) in the absence or presence of neutralizing anti-TGF-β1 antibody (0.1 μg/ml), fetuin (10 μg/ml), anti-FGF-2 antibody (4.5 μg/ml), anti-IGF-1 antibody (5 μg/ml), or anti-PDGF antibody (5 μg/ml). Fetuin was used under some conditions because, as a glycoprotein, it can associate with TGF-β1 and prevent TGF-β1 from binding to TGF-β1 receptor (TβR) II and mediating a signal (13). KGF was used because retroviruses transfect only proliferating cells and because it is an AEC mitogen that induces only AEC proliferation in lung explants (10, 14).

Quantitation of TGF-β1 by ELISA—The TGF-β1 present in the conditioned medium was quantitated using an ELISA kit designed to detect only biologically active TGF-β1 that is not associated with lamin-associated polypeptide-1 (10, 15, 16). To determine the quantity of the total TGF-β1 in the sample, an aliquot of the conditioned media was acidified to remove latency-associated polypeptide-1 from any latent TGF-β1 (L-TGF-β1) present in the conditioned medium. The same conditioned medium was neutralized and used in the ELISA to determine the total TGF-β1 present in the sample.
Preparation of Fibroblast Cultures—In the explant model, the release of TGF-β1 in the conditioned medium overlying the explants was detected in maximal quantities 7 days after transfection, but was barely detectable 14 days after transfection and was not detected in the conditioned medium 21 and 28 days after transfection (10). After 14 days, the explant lung tissue demonstrated maximal fibroblast and myofibroblast numbers and connective tissue synthesis, suggesting that, 14 days after the release of TGF-β1, there is a maximal expansion of fibroblasts (10). For this reason, it was expected that the isolation of fibroblasts from the explant 14 days after transfection of AECs was likely to be the ideal time interval from the release of TGF-β1 to obtain fibroblasts. 14 days after transfection, the explanted lung slices were cut manually into small pieces (~0.5 mm³), placed on culture dishes, and cultured with DMEM in the presence of 10% fetal calf serum, 100 unit/ml penicillin, and 100 μg/ml streptomycin. Fibroblasts were observed to grow out from the explant 7 days later. At this time, the pieces of lung explant were discarded, and the fibroblasts were obtained by trypsinization and plated in equal numbers on culture dishes for 6 weeks. The control fibroblasts were obtained from lung explants cultured in medium, pMX with no TGF-β1 insert, or KGF. In some instances, fibroblasts were obtained from explants transfected with pMX-L-s223,225-TGF-β1 but also cultured with fetuin (10 μg/ml) or antibody to TGF-β1 (0.1 μg/ml), FGF-2 (4.5 μg/ml), IGF-1 (5 μg/ml), or PDGF (5 μg/ml). For some experiments, fibroblasts from untreated explants were cultured with TGF-β1 (10 ng/ml) in the absence or presence of anti-FGF-2 (4.5 μg/ml), anti-IGF-1 (5 μg/ml), or anti-PDGF (5 μg/ml) antibody or an inhibitor of MAPK activation (SB203580 (10 μM), PD98059 (40 μM), or SP60025 (20 μM)). The fibroblasts were then used for [³H]thymidine incorporation or Western analysis.

Vimentin Staining of Rat Lung Tissue and Fibroblasts in Culture—Rat lung tissue pieces and/or fibroblasts were fixed in −10 °C methanol. After air-drying, the cells and/or lung sections were incubated with 3% H₂O₂ in methanol and then washed twice with Tris-buffered saline (TBS)/TWEEN. The slides were incubated with universal blocking solution and incubated with anti-vimentin antibody (1:80 dilution) for 30 min. The slides were washed twice with TBS/TWEEN, incubated with biotinylated, rat-absorbed rabbit anti-mouse antibody (1:300 dilution) for 20 min, and rinsed in TBS/TWEEN. Horseradish peroxidase-conjugated streptavidin was applied to lung tissue pieces and/or fibroblasts for 20 min, washed with TBS/TWEEN, and incubated with 3,3’-diaminobenzidine chromogen for 5 min. The slides were rinsed in distilled water, counterstained with hematoxylin or stained with hematoxylin and eosin, and mounted in aqueous permanent mounting medium.

Extraction and Quantitation of FGF-2—To release and quantify extracellular FGF-2 in fibroblast cultures, the fibroblasts were washed with DMEM and then incubated with 0.25 ml of 20 mM Tris-HCl (pH 7.2) and 2 M NaCl for 2 min (17). The FGF-2 in this medium was quantitated using an FGF-2 detection ELISA kit (R&D Systems) according to the manufacturer’s instructions. The fibroblasts remaining were washed with DMEM, lysed with triple-detergent lysis buffer (50 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, and 5 mg/ml sodium deoxycholate) in the presence of 1 μg/ml aprotinin, 1 μg/ml leupeptin, 5 μg/ml pepstatin A, 1 mM PMSF, and phosphatase inhibitors (1 mM NaF and 1 mM Na₃VO₄) (all from Sigma). Cell lysates were centrifuged at 12,000 rpm for 20 min, and the supernatant was used to quantify FGF-2 in the same ELISA as described above.

[³H]Thymidine Incorporation—To determine fibroblast DNA synthesis, [³H]thymidine incorporation was used. 6 weeks after culture, the fibroblasts were cultured in serum-free DMEM cell concentration of 2 × 10⁴/ml for 24 h. [³H]Thymidine was added to the cell cultures at 0.5 μCi/ml for 24 h, and the fibroblasts were harvested. The degradations/min were measured and used as an index of DNA synthesis and fibroblast proliferation.

Expression of c-Myc, p15INK46, p21CIP1, or p27KIP by Fibroblast Cell Lines and L2 Cells—To determine whether fibroblasts other than primary interstitial fibroblasts respond to TGF-β1 by regulation of c-Myc, p15INK46, p21CIP1, or p27KIP, C3H/10T1/2 and NIH/3T3 fibroblasts were cultured with TGF-β1 in the absence or presence of neutralizing anti-FGF-2 antibody. Because the regulation of c-Myc, p15INK46, p21CIP1, or p27KIP has classically been described for epithelial cells, L2 cells, an AEC line (18), were cultured with TGF-β1 in the absence or presence of neutralizing anti-FGF-2 antibody in the same manner and used to determine the expression of c-Myc, p15INK46, p21CIP1, or p27KIP, and the results were compared with those obtained with the fibroblast cell lines.

Western Analysis to Detect and Quantitate Total and Phosphorylated p38 MAPK, ERK1/2, and JNK and c-Myc, p15INK46, p21CIP1, or p27KIP—The fibroblasts remaining after collection of the conditioned medium were washed with phosphate-buffered saline and detached by trypsinization (10). Whole cell protein was extracted with triple-detergent lysis buffer. The protein levels of each sample were determined using a Bio-Rad protein assay. Samples of protein (25 μg) were electrophoresed on a 10% SDS-polyacrylamide gel in a Mini-ProteanII electrophoresis cell (Bio-Rad). Protein molecular weight markers (Amer sham Biosciences) were run parallel to each blot as an indicator of the molecular weight. Equality of protein loading was evaluated as described (10) using silver stain (data not shown), Ponceau S staining solution (Sigma) (data not shown), or Coomassie Brilliant Blue (Sigma) (data not shown). In some instances, equality of protein loading, cell viability, and activity were confirmed by immunoblotting with anti-β-actin antibody (19). The separated proteins were transferred onto nitrocellulose membrane (Innovigen) in a Mini Trans-Blot chamber with transfer buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol). The nitrocellulose membrane was blocked with 5% instant skim milk in TBS. For detection of total and phosphorylated p38 MAPK, a 1:1000 dilution of antibody was used; for p15INK46, a 1:500 dilution of antibody was used; for p21CIP1, a 1:500 dilution of antibody was used; and for p27KIP, a 1:3000 dilution of antibody was used for β-actin.

As recommended by the manufacturer, the nitrocellulose membrane was washed and incubated with horseradish peroxidase-linked secondary antibody (anti-rabbit or anti-goat IgG; Bio-Rad) (10, 15, 16). The washed blots were exposed to an enhanced chemiluminescence detection system (ECL, Amersham Biosciences) and recorded on an autoradiograph (Eastman Kodak X-Omat film) (10, 15, 16). Prior to reprobing, the same nitrocellulose membrane was incubated at 50 °C for 30 min with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 0.5 mM Tris-HCl (pH 6.7)) (10). After a rinse in TBS, the membranes were incubated with the ECL detection reagents and exposed to Kodak film (23, 28, 29). If no band was detected, the same nitrocellulose membrane was blocked using 5% instant skim milk in TBS, followed by incubation with the primary and secondary antibodies as described (10, 15, 16).

Effects of MAPK Inhibitors on Fibroblast Proliferation—To confirm that induction of phosphorylated p38 MAPK, JNK, or ERK1/2 regulates cell proliferation, the fibroblasts were cultured with SB203580 (10 μM; an inhibitor of phosphorylated p38 MAPK) (20), PD98059 (40 μM; an inhibitor of ERK1/2) (21, 22), or SP60025 (20 μM; an inhibitor of JNK) (36) for 30 min prior to the addition of TGF-β1 (10 ng/ml). This was followed by [³H]thymidine incorporation (37), whereas separate cell cultures maintained under the identical conditions were used for Western analysis to confirm the inhibition of expression of phosphorylated...
p38 MAPK, ERK1/2, and JNK by the presence of the respective antagonists.

RESULTS

Fibroblast Phenotyping—Minced lung pieces from explants were examined after 1 week in culture. Lung pieces from explants that received no treatment, KGF only, or pMX had minimal numbers of fibroblasts growing from the tissue (Fig. 1, A–C). The first evidence of heightened fibroblast proliferation was noted when lung tissue from explants with successful transfection of AECs with pMX-L-s223,225-TGF-β1 (but not controls) showed outgrowth of fibroblasts from the minced explanted tissue in culture (Fig. 1D). Vimentin stains mesenchymal cells (23). Alveolar macrophages, which are of hematopoietic lineage, were used as a negative control for vimentin and did not stain with vimentin (magnification ×20). F, the fibroblasts were from explants cultured in medium and represent the staining observed in fibroblast cultures under all conditions. All cells were positive for vimentin and confirmed the phenotype of cells in culture to be fibroblasts. The figure represents results from experiments done four times.

Vimentin staining of fibroblasts from lung explants. Normal rat lungs infused with agarose were sliced into 5-mm-thick sections and cultured for 14 days in medium (A), KGF (B), pMX with no TGF-β1 insert (C), or pMX-L-s223,225-TGF-β1 and KGF (D) prior to mincing the lung tissue for culture. 1 week later, the explants were stained with hematoxylin and eosin. A–C, a few scattered fibroblasts are seen to grow from lung tissue in culture (magnification ×20). D, Fibroblasts from pMX-L-s223,225-TGF-β1- and KGF-treated explants grew in a dense manner from the tissue (magnification ×20) as early as 1 week in culture. The figure represents results from experiments done four times.

FIGURE 1. Vimentin staining of fibroblasts from lung explants. Normal rat lungs infused with agarose were sliced into 5-mm-thick sections and cultured for 14 days in medium (A), KGF (B), pMX with no TGF-β1 insert (C), or pMX-L-s223,225-TGF-β1 and KGF (D) prior to mincing the lung tissue for culture. 1 week later, the explants were stained with hematoxylin and eosin. A–C, a few scattered fibroblasts are seen to grow from lung tissue in culture (magnification ×20). D, Fibroblasts from pMX-L-s223,225-TGF-β1- and KGF-treated explants grew in a dense manner from the tissue (magnification ×20) as early as 1 week in culture. The figure represents results from experiments done four times.

TGF-β1 Release by Fibroblasts in Culture—After 6 weeks, there was no evidence of active TGF-β1 in the conditioned medium of fibroblasts obtained from pure cell preparations under the various explant conditions (TABLE ONE). However, latent TGF-β1 was found in the conditioned medium of fibroblasts from explants with successful transfection with pMX-L-s223,225-TGF-β1, which was particularly high when fetuin had been present in the initial explant cultures (TABLE ONE). The reason for the increase in L-TGF-β1 in the conditioned medium of fibroblasts from explants treated with pMX is unclear at present. Latent TGF-β1 is biologically inactive (7–10). The release of L-TGF-β1 by the fibroblasts could be a nonspecific response to pMX; and because L-TGF-β1 has no biological activity, it is unlikely to regulate proliferation of fibroblasts in these experiments. These findings suggest that the induction of fibroblast proliferation is an effect of the release of active TGF-β1 by AECs 6–7 weeks earlier and not of the release of active TGF-β1 by the fibroblasts themselves while they were maintained in culture.

Fibroblast Proliferation—[3H]Thymidine incorporation into fibroblasts from explants with successful transfection of AECs with pMX-L-s223,225-TGF-β1 compared with fibroblasts from explants cultured with medium, pMX, KGF, or pMX plus KGF had a 6.59 ± 1.65-fold (mean ± S.E.) increase in [3H]thymidine incorporation, an index of DNA synthesis and a marker of cell proliferation (Fig. 2). Fibroblasts from explants with AECs transfected with pMX-L-s223,225-TGF-β1 but cultured with fetuin, the TGF-β homolog, or neutralizing antibody to TGF-β1 compared with fibroblasts from pMX-L-s223,225-TGF-β1-treated explants had a decrease in [3H]thymidine incorporation, which was not statistically different from the [3H]thymidine incorporation into fibroblasts from explants that received no treatment. Overall, these data demonstrate that, when AECs of lung explants release active TGF-
IGF-1, and PDGF or the regulation of receptors for FGF-2, IGF-1, and transfected explants (body.) [3H]Thymidine incorporation into fibroblasts from explants cultured with pMX-L-s223,225-TGF-β1 was also cultured with fetuin (10 μg/ml) (first bar), anti-FGF-2 antibody (10 μg/ml) (second bar), anti-IGF-1 antibody (10 μg/ml) (third bar), anti-PDGF antibody (10 μg/ml) (fourth bar), KGF (25 ng/ml) (fifth bar), or pMX (10^6 TCID₅₀) plus KGF (25 ng/ml) (sixth bar) or after successful transfection of AECs with pMX-L-s223,225-TGF-β1 (10^6 TCID₅₀) (seventh bar) were used. Some explants successfully transfected with pMX-L-s223,225-TGF-β1 were also cultured with fetuin (10 μg/ml), anti-FGF-2 antibody (10 μg/ml) (eighth bar), anti-IGF-1 antibody (10 μg/ml) (ninth bar), or anti-PDGF antibody (10 μg/ml) (tenth bar). [3H]Thymidine (3H-TdR) incorporation is presented as -fold above control value. Fibroblasts obtained from explants with successful transfection of AECs with pMX-L-s223,225-TGF-β1 had a >6-fold increase in [3H]thymidine incorporation, whereas neutralizing anti-TGF-β1 antibody, anti-FGF-2 antibody, or fetuin decreased [3H]thymidine incorporation. *, p < 0.01 for [3H]thymidine incorporation into fibroblasts obtained from pMX-L-s223,225-TGF-β1-transfected explants compared with fibroblasts obtained from untreated explants. [3H]Thymidine incorporation into fibroblasts from explants cultured with pMX, KGF, pMX-L-s223,225-TGF-β1, and anti-TGF-β1 antibody or fetuin was not significant compared with fibroblasts obtained from untreated controls. Explants with AECs successfully transfected with pMX-L-s223,225-TGF-β1 cultured with antibodies to FGF-2, IGF-1, and PDGF had reduced [3H]thymidine incorporation. The most profound inhibition of DNA synthesis was in the presence of anti-FGF-2 antibody. **, p < 0.001 for [3H]thymidine incorporation into fibroblasts obtained from pMX-L-s223,225-TGF-β1-transfected explants compared with fibroblasts obtained from TGF-β1-transfected explants in the presence of anti-FGF-2 antibody. [3H]Thymidine incorporation into fibroblasts from explants cultured with pMX-L-s223,225-TGF-β1 and antibody to IGF-1 or PDGF compared with fibroblasts obtained from transfected explants (fifth bar) was not significant. Statistical analysis was done using Wilcoxon’s rank sum test. The means ± S.E. from experiments done four times are shown.

Role of Growth Factors in the Proliferation of Fibroblasts—It has previously been reported in a variety of cell culture systems that the proliferative effects of TGF-β1 on cells of the mesenchymal lineage are indirect by the induction and release of other cytokines such as FGF-2, IGF-1, and PDGF or the regulation of receptors for FGF-2, IGF-1, and PDGF (24–32). We next determined the role of FGF-2, IGF-1, and PDGF in the TGF-β1-mediated proliferation of pulmonary fibroblasts. During the time of transfection with pMX-L-s223,225-TGF-β1, some of the explants were cultured in the absence or presence of antibody to FGF-2, IGF-1, or PDGF for 2 weeks. Neutralizing antibody to FGF-2 present at the time of transfection totally abrogated the TGF-β1-mediated [3H]thymidine incorporation into fibroblasts (Fig. 2). Neutralizing antibodies to PDGF and IGF-1 present at the time of transfection reduced [3H]thymidine incorporation into isolated fibroblasts by 3.01 ± 0.95 and 2.33 ± 0.67-fold, respectively (Fig. 2). These findings suggest that TGF-β1-induced [3H]thymidine incorporation and thus fibroblast proliferation in this model are mediated primarily by FGF-2 and that the effect commences at the time of release of TGF-β1 by AECs.

Expression of MAPKs by Fibroblasts—An important pathway activated by mitogens and growth factors is phosphorylation of MAPKs p38 MAPK, JNK, and ERK1/2 (32–35). Phosphorylation at very specific sites of these MAPKs activates the MAPKs, which can be detected by antibodies to phosphorylated p38 MAPK, JNK, and ERK1/2 (20–22). Fibroblasts obtained from explants with successful transfection of AECs with pMX-L-s223,225-TGF-β1 did not express phosphorylated p38 MAPK, ERK1/2, or JNK (data not shown). Phosphorylation of MAPK may be an early effect of a mitogen on fibroblasts and therefore not observed in these fibroblasts, which were 6–7 weeks remote from the time of release of TGF-β1 by AECs. To determine whether phosphorylation of MAPK by primary lung fibroblasts in response to TGF-β1 occurs at an earlier time of exposure to TGF-β1, fibroblasts from untreated lung explants were cultured with TGF-β1 for 1, 5, and 60 min. Western analysis of the expression of phosphorylated p38 MAPK, ERK1/2, and JNK demonstrated that quiescent fibroblasts showed little or no signal, but after incubation with TGF-β1, phosphorylation of p38 MAPK and JNK (but not ERK1/2) was induced (Fig. 3). We next determined whether the induction of MAPKs by TGF-β1 is due indirectly to actions of a fibroblast mitogen by culturing the fibroblasts with TGF-β1 concomitantly with antibody to FGF-2, IGF-1, or PDGF. Phosphorylated p38 MAPK and JNK were dramatically abrogated when antibody to FGF-2 was cultured with TGF-β1 at all time intervals (Fig. 3). Phosphorylated ERK1/2 was not affected by the presence of antibody to FGF-2 (see Fig. 5). Fibroblasts showed constitutive expression of total p38 MAPK, JNK, and ERK1/2, which was not affected by serum starvation or treatment with TGF-β1 in the absence or presence of anti-FGF-2 antibody (Fig. 3) or anti-IGF-1 or anti-PDGF antibody (data not shown). Ponceau S and silver staining (data not shown) confirmed equal loading of proteins. β-Actin and total p38 MAPK, JNK, and ERK1/2 were also equivalent in all lanes, demonstrating equality in loading and confirming that the fibroblasts in the absence or presence of TGF-β1 with or without anti-FGF-2 antibody were viable, active, and able to synthesize proteins (Fig. 3). Antibodies to IGF-1 and PDGF had no effect on phosphorylation of the MAPKs (data not shown).

Release of FGF-2—Because the effects of TGF-β1 on phosphorylation of JNK and p38 MAPK were so immediate and seemingly mediated by FGF-2, the findings suggest that the effects of FGF-2 are likely due to the
release of a previously synthesized and stored pool of FGF-2. FGF-2 generated by fibroblasts localizes to the ECM and can be released from the ECM by the actions of 2 M NaCl (26, 36). Treatment of fibroblasts cultures with 2 M NaCl resulted in a pronounced increase in the quantity of FGF-2 when the fibroblasts had been treated with TGF-β1 compared with fibroblasts not treated with TGF-β1 (Fig. 4). Supernatants of fibroblast lysates representing intracellular FGF-2 were not affected by the absence or presence of TGF-β1 (data not shown).

**FGF-2 Phosphorylation of MAPKs**—Last, further confirming that FGF-2 induces phosphorylation of MAPKs, fibroblasts cultured with FGF-2 (2.5 ng/ml) demonstrated an increase in phosphorylation of these MAPKs (Fig. 5). To determine whether expression of phosphorylated p38 MAPK, ERK1/2, or JNK is important for fibroblast proliferation, the fibroblasts were cultured for 5 min (data not shown) or 30 min with SB203580 (a well described inhibitor of p38 MAPK phosphorylation) (20), PD98059 (an inhibitor of phosphorylation ERK1/2) (21, 22), or SP60025 (an inhibitor of JNK phosphorylation) (35, 50) prior to the addition of TGF-β1. The presence of all inhibitors diminished [³H]thymidine incorporation (Fig. 6) and expression of the phosphorylated form of all three MAPKs at both time intervals. Expression of total MAPKs was not altered. p = 0.03–0.04 for -fold increase in phosphorylated MAPKs compared with untreated controls. Statistical analysis was done using Wilcoxon’s rank sum test. The means ± S.E. from experiments done four times are shown.
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antibody to FGF-2 did not decrease ERK1/2 phosphorylation (Fig. 3). However, because anti-FGF-2 antibody abrogated phosphorylated p38 MAPK and JNK, the phosphorylation of which is important for DNA synthesis (Fig. 6), the findings suggest that, in this model, the TGF-β1-induced proliferation of fibroblasts mediated by FGF-2 is through phosphorylation of p38 MAPK and JNK, but not ERK1/2.

Anti-proliferative Response of Fibroblasts to AEC-derived TGF-β1—TGF-β1 is a potent inhibitor of proliferation for most phenotypes of cells (38). The response of cells to the anti-proliferative effects of TGF-β1 is characterized by suppression of c-Myc and induction of CKIs p15INK46, p21CIP1, and p27KIP (39–41). To determine whether primary fibroblasts respond to the anti-proliferative effects of TGF-β1, the expression of c-Myc, p15INK46, p21CIP1, or p27KIP was determined. Fibroblasts from explants with successful pMX-L-s223,225-TGF-β1 transfection of AECs did express c-Myc, p15INK46, p21CIP1, or p27KIP (data not shown). The changes in expression of c-Myc, p15INK46, p21CIP1, or p27KIP may be an early response to TGF-β1, whereas the fibroblasts used in these experiments were 6–7 weeks remote from the time of exposure to AEC-derived TGF-β1. For this reason, primary lung fibroblasts obtained from untreated rat lung explants were cultured with TGF-β1 for 5 and 60 min. They did not respond to TGF-β1 by changes in expression of c-Myc, and p15INK46, p21CIP1, and p27KIP were not expressed (data not shown), as described previously for epithelial cells (52–54).

To determine whether the lack of response by fibroblasts to TGF-β1 by induction of p15INK46, p21CIP1, or p27KIP is a feature of all fibroblasts, two cell lines of fibroblasts (C3H/10T1/2 and NIH/3T3) were cultured with TGF-β1 in the same manner as the primary lung fibroblasts. NIH/3T3 fibroblasts expressed c-Myc, p15INK46, p21CIP1, and p27KIP. C3H/10T1/2 fibroblasts expressed c-Myc, p21CIP1, p27KIP, and p15INK46 (Fig. 8, A and B). The expression of c-Myc, p21CIP1, or p27KIP did not change in the presence of TGF-β1 (Fig. 8, A and B). Unlike in the primary fibroblasts from the lung explants or cell lines of fibroblasts, the L2 cells (a rat AEC line) expressed c-Myc, and when cultured with TGF-β1, there was a decrease in c-Myc expression (Fig. 8, A and B). In addition, p21CIP1 and p27KIP were barely detectable in L2 cells; but after incubation with TGF-β1 for 5 and 60 min, p21CIP1 and p27KIP were induced (Fig. 8A). L2 cells did not express p15INK46 in the presence of TGF-β1. Because the presence of TGF-β1 in cultures of fibroblasts caused a release of FGF-2 (Figs. 2–4), it was possible that FGF-2 interfered with the regulation of expression of c-Myc and CKIs p15INK46, p21CIP1, and p27KIP. However, when L2 cells and C3H/10T1/2 and NIH/3T3 fibroblasts were cultured with TGF-β1 in the presence of neutralizing antibody to FGF-2, there was no effect on the expression of c-Myc and CKIs p15INK46, p21CIP1, and p27KIP (Fig. 8B). These findings suggest that the expression of c-Myc and CKIs p15INK46, p21CIP1, and p27KIP by primary lung fibroblasts and perhaps fibroblasts in general is not affected by the presence of TGF-β1 or FGF-2.

DISCUSSION

In a recently developed model that resembles IPF, the most significant event is the release of biologically active TGF-β1 (10). Despite being an anti-proliferative agent, TGF-β1 either directly or indirectly induces the proliferation of fibroblasts beneath the AECs and in the pulmonary interstitium. Anti-FGF-2 antibody present in lung explants at the time of release of AEC-derived TGF-β1 inhibits the development of a proliferative fibroblast phenotype, suggesting that TGF-β1 regulation of pulmonary interstitial fibroblast proliferation is mediated by FGF-2 and occurs at the time of release of TGF-β1 when the explant is
intact. Furthermore, AEC-derived TGF-β1 results in fibroblasts that retain a heightened proliferative state up to 6 weeks after exposure to TGF-β1.

FGF-2 regulates proliferation by interacting with its receptor and inducing mitogenesis via the MAPKs (42–45). Phosphorylation of ERK1/2 has been reported to be important for mitogen-mediated proliferation. The results from the present study are therefore unique in that antibody to FGF-2 totally abrogated phosphorylation of p38 MAPK and JNK, but not ERK1/2, which was constitutively phosphorylated. This suggests that the quantities of FGF-2 released by the presence of TGF-β1 induce proliferation of primary interstitial lung fibroblasts through phosphorylation of p38 MAPK and JNK, but not ERK1/2.

Fibroblasts treated with TGF-β1 take up to 2 h before there is evidence of FGF-2 protein (43). We demonstrated that incubation of fibroblasts with TGF-β1 for as little as 1 min could increase the quantity of FGF-2 released from fibroblasts without affecting intracellular FGF-2. The rapidity of these effects suggests that TGF-β1 induces the release of preformed FGF-2. Up to 30% of FGF-2 synthesized by a fibroblast may be associated with the ECM surrounding the fibroblast (46). Because the presence of active TGF-β1 does not affect intracellular FGF-2, the findings suggest that a pool of FGF-2 exists extracellularly and can be released by TGF-β1. ECM-associated FGF-2 can be released by the actions of proteases such as plasmin (47) and heparinase (48). TGF-β1 inhibits the generation of plasmin (49), so it is improbable that the action of plasmin results in the release of FGF-2. It is unknown if TGF-β1 induces fibroblasts to release heparinase, which could in turn release FGF-2 from the ECM. FGF-2 associated with the ECM or cell membrane can also be released by mechanical strain or stretch (50). TGF-β1 causes contraction of fibroblasts (51), and the shear stresses created by contraction may release FGF-2 in the current model. Once released from the ECM, FGF-2 has been found to be a potent mitogen for fibroblasts (52), which was evident in our findings. Another mechanism of release of FGF-2 may be by leakage of FGF-2 from the cell upon cell death or by increase in membrane permeability from sublethal damage to a cell (53–55). Recently, shedding of membrane vesicles containing FGF-2 has been described as a mechanism of release of intracellular FGF-2 (56). It is conceivable that TGF-β1 augments vesicular release of FGF-2 from these fibroblasts but is unlikely because there is no definitive evidence that TGF-β1 affects vesicle trafficking. There was no evidence of fibroblast injury or death in the current model, as the fibroblasts synthesized β-actin in identical quantities in the absence or presence of TGF-β1 with or without anti-FGF-2 antibody. Further studies are required to determine the mechanism by which TGF-β1 leads to the release of FGF-2 by fibroblasts in this model. In this study, quiescent fibroblasts responded to TGF-β1-induced effects of FGF-2 on phosphorylation of p38 MAPK and JNK within 1, 5, and 60 min. The rapidity with which phosphorylation of p38 MAPK and JNK were detected suggests that FGF-2 phosphorylates pre-existing p38 MAPK and JNK rather than inducing the synthesis and subsequent phosphorylation of these proteins. Because large quantities of total p38 MAPK, JNK, and ERK1/2 were present in fibroblasts irrespective of treatment with TGF-β1, the findings suggest the possibility that the TGF-β1-
mediated release of FGF-2 induces phosphorylation of pre-existing p38 MAPK and JNK.

In some instances, TGF-β1 has been reported to regulate phosphorylation of MAPKs (57, 58). However, it has never been clearly demonstrated whether the regulation of MAPKs by TGF-β1 is a direct effect of TGF-β1 or an indirect effect of TGF-β1 mediated by the release of another cytokine (57, 58). Our observations demonstrate that TGF-β1 leads to phosphorylation of MAPKs by the release of FGF-2 from TGF-β1-stimulated fibroblasts. In view of the present findings, the mechanisms by which TGF-β1 leads to MAPK phosphorylation reported in previous studies may need to be re-evaluated.

TGF-β1 is well recognized as a potent inhibitor of cell proliferation of epithelial, endothelial, and hematopoietic cells (57, 58). Signal transduction mediated by TGF-β resulting in the inhibition of proliferation starts when TGF-β1 binds to TβR-II on the cell membrane (57, 58). TβR-II is constitutively phosphorylated; and after interacting with TGF-β1, TβR-I is then phosphorylated by TβR-II at the serine and threonine residues located in the glycine-serine-rich domain of TβR-I (57). Once TβR-I is phosphorylated, the protein targets of activated TβR-I are membrane-associated intracellular mediators of the Smad family (57). The receptor-regulated Smad2 and Smad3 proteins are phosphorylated by TβR-I at a C-terminal SXSX motif (57). Once phosphorylated, the receptor-regulated Smads form a complex with common partner Smad4, which translocates to the nucleus (57).

In association with other transcription factors, these Smad proteins activate the transcription of a number of target genes (57). Regarding the regulation of cell proliferation, the TGF-β-activated Smad complexes suppress transactivation of c-Myc, a key regulator of cell proliferation (39, 41), c-Myc suppresses expression of growth arrest genes and CKIs p15INK4a, p21CIP1, and p27KIP. In the presence of TGF-β1, there is increased expression of p15INK4a, p21CIP1, or p27KIP (39–41), resulting in growth arrest of the cell at G1. This schema of cell cycle arrest has been described in epithelial cell lines (39–41, 57, 58).

Primary pulmonary fibroblasts cultured with TGF-β1 did not express c-Myc, p15INK4a, p21CIP1, or p27KIP. Further confirmation that fibroblasts do not respond to TGF-β1 by regulation of c-Myc, p15INK4a, p21CIP1, or p27KIP was obtained when the fibroblasts cell lines 10T1/2 and NIH/3T3 cultured with TGF-β1 also did not respond by changes in expression of c-Myc, p15INK4a, p21CIP1, or p27KIP. When L2 cells were cultured with TGF-β1, there was a decrease in c-Myc expression, whereas there was an increase in p21CIP1 and p27KIP expression. Although TGF-β1 may lead to induction of p15INK4a, p21CIP1, or p27KIP, this response is dependent on the cell type. The regulation of p15INK4a by TGF-β1 appears to be consistently observed in mammary epithelial cells, but not necessarily all epithelial cells. Despite the lack of expression of p15INK4a by L2 cells, TGF-β1 suppressed c-Myc and induced p21CIP1 and p27KIP, which is characteristic of the epithelial cell response to TGF-β1. The expression of c-Myc and the CKIs was totally lacking in primary lung fibroblasts, and the expression of c-Myc and the CKIs in fibroblast cell lines was not altered when they were cultured with TGF-β1 in either the absence or presence of anti-FGF-2 antibody. These findings demonstrate for the first time that fibroblasts do not express the well-documented changes in c-Myc and CKIs important for TGF-β1-mediated inhibition of proliferation. We have not investigated any changes in expression of c-Myc, p15INK4a, p21CIP1, or p27KIP at the mRNA level, but this may be of questionable relevance if the fibroblasts do not respond by changes in protein expression of these CKIs necessary for cell cycle arrest.

Collectively, these findings suggest that the release of TGF-β1 by AECs induces interstitial pulmonary fibroblasts to proliferate by the actions of FGF-2, whereby phosphorylation of p38 MAPK and JNK is critical in TGF-β1-induced fibroblast proliferation. Furthermore, fibroblasts do not appear to respond to the anti-proliferative effects of TGF-β1. The findings reported in this study and those of an earlier study showing remodeling of the lung parenchyma by AEC-derived TGF-β1 (10) suggest that AEC-derived TGF-β1 can result in marked proliferation of fibroblasts that is rapid in onset and long lasting. The clinical implications of our findings are highly significant. It is now speculated that the regulation of inflammatory cells in the pathogenesis of IPF is minimal or of secondary importance (10, 59, 60). However, the expansion of fibroblasts and the development of fibroblast foci may be critical to the pathogenesis and progressive nature of IPF (1, 5, 6). If, for example, in IPF, the release of TGF-β1 by AECs on each occasion results in a similar burst of fibroblast proliferation that lasts 6 weeks or longer, then repeated release of TGF-β1 by AECs would rapidly affect fibroblast proliferation and connective tissue synthesis that could last for months. These biological events in vivo would explain the progressive nature of IPF. Because fibroblast proliferation and remodeling occur in the absence of inflammatory cells, these results offer important insight into the lack of therapeutic efficacy of immunosuppressive agents for the treatment of IPF.

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