Interleukin-7 and Transforming Growth Factor-β Play Counter-regulatory Roles in Protein Kinase C-δ-dependent Control of Fibroblast Collagen Synthesis in Pulmonary Fibrosis*

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Transforming growth factor-β (TGF-β) is a potent fibrogenic factor responsible for promoting synthesis of extracellular matrix. Interleukin-7 (IL-7) inhibits TGF-β signaling by up-regulating Smad7, a major inhibitor of the Smad family. In a variety of cells, TGF-β-mediated activation of target genes requires active protein kinase C-δ (PKC-δ) in addition to Smads (1). We determined the role of PKC-δ in the regulation of pulmonary fibroblast collagen synthesis in response to TGF-β and IL-7 stimulation. Here we show that TGF-β and IL-7 have opposing effects on PKC-δ. TGF-β stimulates, while IL-7 inhibits, PKC-δ activity. IL-7 inhibits TGF-β-induced PKC-δ phosphorylation at Ser-645 and Thr-505. Inhibition of PKC-δ with specific small inhibitory RNA restores TGF-β-mediated induction of Smad7 and in parallel significantly reduces TGF-β-mediated collagen synthesis. Thus, PKC-δ may play a critical role in the pathogenesis of pulmonary fibrosis and may serve as a molecular target for therapeutic intervention to suppress fibrosis.

Pulmonary fibrosis is characterized by disturbances of extracellular matrix protein deposition resulting from fibroblast activation and proliferation (2). TGF-β is a major driving force in the pathogenesis of pulmonary fibrosis (2). Overexpression of TGF-β1 in pulmonary fibrosis contributes to the dysregulation of normal homeostatic mechanisms at multiple levels; it enhances synthesis and deposition of extracellular matrix components including collagen and can also alter the balance of matrix metalloproteinases and their inhibitors (2, 3). TGF-β signaling and regulation are mediated by a family of Smads, TGF-β signaling through the induction of Smad7 (5). In addition to Smads, TGF-β requires active PKC-δ to activate its target genes in a variety of cells including pulmonary fibroblasts (1). PKC-δ, a ubiquitously expressed kinase involved in a variety of cellular signaling pathways, has been implicated in de novo synthesis of extracellular matrix components (6). PKC-δ participates in the up-regulation of type I (COL1A1) and type III (COL3A1) collagen gene expression mediated by TGF-β in scleroderma fibroblasts (7). Based on the importance of PKC-δ in TGF-β signaling, we determined whether PKC-δ modulated collagen synthesis in response to TGF-β and IL-7 stimulation in pulmonary fibroblasts.

MATERIALS AND METHODS

Cell Culture—Isolation, passage, and specific cell marker characterization of human pulmonary fibroblasts were performed as described previously (5). Fibroblasts obtained from patients with idiopathic pulmonary fibrosis (PPF) or non-fibrotic lung diseases (NF) were maintained in 5% CO2 in air as monolayers at 37 °C in 75-cm² tissue culture flasks containing 20 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine (JRH Biosciences, Lenexa, KS).

Cytokines and Antibodies—Human recombinant TGF-β1 (3.2 × 10⁶ units/μg) and recombinant human IL-7 (10⁶ units/μg) were obtained from R & D Systems, Minneapolis, MN. Rabbit anti-PKC-δ, rabbit anti-PKC-δ (Ser645), and rabbit anti-PKC-δ (Thr505) antibodies were obtained from BIOSOURCE International, Inc., Camarillo, CA. Rabbit anti-PKC-δ (pThr505) antibody was obtained from Cell Signaling Technology, Inc., Beverly, MA. Lyophilized human recombinant TGF-β1 was reconstructed in sterile 4 ml HCl containing 0.1% bovine serum albumin in a final concentration of 10 μg/ml and stored at −70 °C in aliquots.

Northern Blot Analysis—High quality total RNA from 10⁶ cells of NF and PPF was prepared by the Trizol method (Invitrogen). Northern blotting was performed as previously described (8). We used the following unique 30-mer oligonucleotide probes for the detection of human COL1A1 and COL3A1 gene expression: 5′-CAGTTCTTGGTCTCGTCA-CAGATCACGTCA-3′ (human COL1A1 probe, corresponding to the coding region 332–361) and 5′-TTCCATTTTCTCCTGGAGGACCACCTG-3′ (human COL3A1 probe, corresponding to the coding region 2051–2080). Duplicate filters were hybridized with 32P-radiolabeled human β-actin cDNA probe serving as controls for equal loading and transfer. The filters were exposed to an x-ray film for documentation and analyzed using phosphor imaging equipment (Molecular Imager, Bio-Rad).

This paper is available online at http://www.jbc.org

Received for publication, March 16, 2004, and in revised form, April 26, 2004
Published, JBC Papers in Press, May 7, 2004, DOI 10.1074/jbc.C400115200
PKC-δ Activity Assays—PFF and NF were incubated in medium alone, in medium containing IL-7 (100 ng/ml), in medium containing TGF-β1 (10 ng/ml), or in medium containing IL-7 and TGF-β1 for 1 h at 37 °C. 5 × 10^5 Cells of each sample were lysed in phosphorylation lysis buffer containing 150 mM NaCl, 200 mM sodium orthovanadate, 10 mM sodium phosphate, 100 mM sodium fluoride, 1.5 mM MgCl₂, 10% glycerol, 1 mM EDTA, 0.5% Triton X-100, and protease inhibitors. Cell lysates were immunoprecipitated with a specific anti-PKC-δ antibody (BIOSOURCE International, Inc.). The immunoprecipitates were washed three times with phosphorylation lysis buffer and two times with kinase buffer, which contains 25 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 20 μg of phosphatidylserine, and 20 mM ATP. The immunoprecipitates were resuspended in 30 μl of kinase buffer containing 5 μg of an exogenous substrate histone H1 and 20 μCi of [γ-32P]ATP and incubated at 25 °C for 30 min. The reaction was terminated by the addition of an equal volume of SDS sample buffer. The PKC-δ activity was determined by the amount of 32P-histone H1 produced as detected by autoradiography following SDS-PAGE electrophoresis.

Western Blot Analysis—PFF and NF under standard growth conditions or in medium supplemented with cytokines were suspended in 1× phosphate-buffered saline buffer containing 1 mM MgCl₂, protease inhibitors (10 μg/ml), and 0.1% Triton X-100. The cell suspension was sonicated and then centrifuged at 11,000 × g for 10 min at 4 °C. Protein content of each sample was determined using the protein assay kit (Bio-Rad). Aliquots containing 80 μg of lysate were boiled for 5 min and electrophoresed on 7.5% polyacrylamide gels. The gels were transferred to Immuno-Blot membranes (Bio-Rad), blocked for 1 h with 5% nonfat milk, and then incubated with specific primary antibodies. The immunoreactive proteins were detected using a Protoblot II AP System kit (Promega Corp., Madison, WI). PKC-δ siRNA—PKC-δ siRNA Duplex1 for the target sequence 5’-AAG ATG AAG GAG GCC CTC AG-3’ was obtained from Qiagen (Valencia, CA). Two micrograms of PKC-δ siRNA were premixed with TransMessenger Transfection Reagent (Qiagen Inc., Valencia, CA) in a ratio of 1:2 to 1.8. PFF and NF were then transfected with PKC-δ siRNA mixture or sorbitol only as controls for 3 h under standard growth conditions.

Real-time RT-PCR—Five hundred nanograms of total RNA from PFF (n = 2) and NF (n = 3) were used for the real-time RT-PCR analysis using unique target gene primers or housekeeping gene β-actin primers serving as controls for equal loading. Primer pairs for the optimal effect on PKC-δ activity in NF (Fig. 2). Thus, IL-7 and TGF-β have opposing effect on PKC-δ activity; IL-7 is inhibitory, while TGF-β is stimulatory, in PFF.

IL-7 Inhibits TGF-β-mediated Phosphorylation of PKC-δ at Thr-505 and Ser-645 in PFF—PKC-δ mediates a wide range of biological responses, and its activity is controlled by distinct phosphorylation sites (9). To determine the characteristic phosphorylation patterns of PKC-δ activation regulated by IL-7 and TGF-β in NF and PFF, we performed Western blot analysis using a panel of specific monoclonal antibodies including anti-total PKC-δ, anti-phospho-PKC-δ [pThr505], anti-phospho-PKC-δ [pTyr311], and anti-phospho-PKC-δ [pSer645] antibodies. PFF and NF were incubated in the following conditions: medium alone, IL-7 (100 ng/ml), TGF-β (10 ng/ml), and IL-7 + TGF-β at 37 °C for 1 h (1, 6). The PKC-δ activity was determined by the amount of [32P]-histone H1 produced as detected by autoradiography following SDS-PAGE electrophoresis. The results show that IL-7 inhibited both constitutive and TGF-β-induced PKC-δ activity in PFF but had no effect on PKC-δ activity in NF (Fig. 2). Therefore, IL-7 and TGF-β have opposing effect on PKC-δ activity; IL-7 is inhibitory, while TGF-β is stimulatory, in PFF.

**RESULTS**

IL-7 Inhibits TGF-β-induced COL1A1 and COL3A1 Gene Expression in PFF—To determine whether IL-7 modulates TGF-β-induced collagen synthesis at the level of regulating COL1A1 and COL3A1 gene expression, PFF were incubated in medium alone, in medium containing acridine-activated recombinant TGF-β1 (10 ng/ml), or in medium containing IL-7 and TGF-β1 for 1 h at 37 °C. 5 × 10^5 Cells of each sample were lysed in phosphorylation lysis buffer containing 150 mM NaCl, 200 mM sodium orthovanadate, 10 mM sodium phosphate, 100 mM sodium fluoride, 1.5 mM MgCl₂, 10% glycerol, 1 mM EDTA, 0.5% Triton X-100, and protease inhibitors. Cell lysates were immunoprecipitated with a specific anti-PKC-δ antibody (BIOSOURCE International, Inc.). The immunoprecipitates were washed three times with phosphorylation lysis buffer and two times with kinase buffer, which contains 25 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, 20 μg of phosphatidylserine, and 20 mM ATP. The immunoprecipitates were resuspended in 30 μl of kinase buffer containing 5 μg of an exogenous substrate histone H1 and 20 μCi of [γ-32P]ATP and incubated at 25 °C for 30 min. The reaction was terminated by the addition of an equal volume of SDS sample buffer. The PKC-δ activity was determined by the amount of 32P-histone H1 produced as detected by autoradiography following SDS-PAGE electrophoresis.

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Inhibition of PKC-δ with Specific siRNA Significantly Increased Smad7 mRNA Levels and, in Parallel, Reduced TGF-β-mediated Collagen Synthesis in PFF—PKC-δ is implicated in de novo synthesis of connective tissue and extracellular matrix components (9). For example, TGF-β-stimulated PKC-δ activity positively regulates Smad transcriptional activity resulting in up-regulation of the COL1A2 gene expression in renal mesangial cells (6). We have shown that IL-7 inhibits TGF-β-mediated
**DISCUSSION**

Idiopathic pulmonary fibrosis (IPF) is a devastating disease with less than a 50% 5-year survival (11). While steroids and other immunosuppressive agents serve as the standard treatment for IPF, these agents are ineffective. The development of new therapeutic paradigms is clearly a clinical priority. The pathogenesis of pulmonary fibrosis includes the deterioration of the normal homeostatic mechanisms regulating the equilibrium between the synthesis and breakdown of the extracellular matrix (2, 3, 11, 12). TGF-β plays a critical role in the pathogenesis of pulmonary fibrosis by enhancing synthesis and deposition of extracellular matrix components including collagen (2, 3). Based on the importance of TGF-β in the pathogenesis of pulmonary fibrosis, we investigated the mechanisms of dysregulation of TGF-β signaling in PFF.

We reported previously that IL-7 has the capacity to block TGF-β signaling by up-regulation of Smad7, a major inhibitory member of the Smad family. *In vitro*, IL-7 inhibits fibroblast collagen deposition in a Smad7-dependent manner (5). *In vivo*, IL-7 limits pulmonary fibrosis (5). Because collagen types I and III are major components of extracellular matrix in pulmonary fibrosis (2, 3), we first investigated the effect of IL-7 on TGF-β-mediated induction of COL1A1 and COL3A1 gene expression in PFF. Consistent with a marked reduction in collagen synthesis as previously reported, the current results show that IL-7 inhibits TGF-β-mediated induction of COL1A1 and COL3A1 gene expression in PFF. Thus, IL-7 inhibits TGF-β-mediated collagen synthesis at both mRNA and protein levels.

PKC-δ is a member of the PKC family of serine-threonine kinases, which play important roles in signaling for various cytokine receptors. Specifically relevant to our current study, the Smad cascade and PKC-δ play a role in the regulation of collagen synthesis in response to TGF-β and IL-7 stimulation in PFF. In preliminary studies, we tested the conditions for optimal gene silencing with PKC-δ siRNA in PFF. As demonstrated by Western blot analysis in Fig. 4A, PKC-δ siRNA, mixed with transfection reagent in a ratio of 1:8, provided an optimal gene silencing effect of PKC-δ (>95% reduction of gene products), while β-actin levels, serving as controls for equal loading, remained unchanged. To determine the role of PKC-δ in Smad7 gene regulation, NF (n = 2) and PFF (n = 3) were preincubated in medium with or without PKC-δ siRNA for 24 h. TGF-β(10 ng/ml) was then added to the cultures, and cells were incubated for additional 90 min prior to isolation of total RNA (10). Smad7 mRNA levels of each sample were determined by real-time RT-PCR analysis. As shown in Fig. 4B, TGF-β significantly increased Smad7 mRNA expression levels up to 16-fold in NF, while TGF-β had no significant effect on Smad7 mRNA levels in PFF (p < 0.01). However, when PFF were incubated with TGF-β plus PKC-δ siRNA, Smad7 mRNA levels only increased 7.3-fold compared with cells incubated with TGF-β alone (p < 0.05). In comparison, inhibition of PKC-δ with specific siRNA in NF did not show significant differences in Smad7 mRNA levels. The results suggest that PKC-δ is important in TGF-β-mediated regulation of Smad7 gene expression in lung fibroblasts. Based on our results and those of others (6, 9), we hypothesized that TGF-β-mediated collagen synthesis in PFF could be blocked by the inhibition of PKC-δ. PFF (n = 3) was cultured in medium alone, in medium containing TGF-β(10 ng/ml), or in medium containing TGF-β plus PKC-δ siRNA for 24 h. The inhibition of PKC-δ with siRNA significantly reduced collagen levels (47–100% reduction) in response to TGF-β stimulation in PFF (Fig. 4C). Thus, PKC-δ may play a critical role in the regulation of fibroblast collagen synthesis in pulmonary fibrosis.
phosphorylated by various tyrosine kinases including Fyn, Src, Lyn, Lck, and Abl (1, 9). Our results show that TGF-β and IL-7 have no effect on PKC-δ phosphorylation at Tyr-311 in both PFF and NF. Thus, Tyr-311 phosphorylation may not be required for PKC-δ activity in human pulmonary fibroblasts. We (5, 14) and others (15–17) have reported marked phenotypic and functional differences between PFF and NF. These differences have been demonstrated at multiple levels including TGF-β receptor expression, expression of cell surface receptors or signal transduction mediators, regulation of TGF-β signaling, and response to cytokines or other stimulators. Normal fibroblasts have the capacity to produce TGF-β in response to a variety of stimuli. This production of TGF-β then serves to control and mediate processes such as wound repair. However, following TGF-β production, regulatory mechanisms must also be initiated to limit TGF-β signaling and avoid, for example, uncontrolled collagen production and fibrogenesis. Thus, exposure of normal cells to TGF-β leads to the rapid induction of Smad7 mRNA expression. In the current study, Smad7 mRNA levels rapidly increased in response to TGF-β stimulation in NF, while no significant differences were observed in PFF. The results suggest that the normal negative feedback regulatory mechanism for TGF-β signaling may be impaired in PFF. Thus, NF are endowed with balanced fibrogenic and antifibrogenic regulatory mechanisms and appear to be resistant to aberrant signals seen in pulmonary fibrosis. In comparison, PFF have a fibrogenic phenotype. Importantly, inhibition of PKC-δ (~95% inhibition) with specific siRNA significantly increased Smad7 mRNA levels in parallel, reduced TGF-β-mediated collagen synthesis in PFF. A, PKC-δ siRNA effectively inhibited PKC-δ as demonstrated by Western blot analysis. Preliminary studies showed that 2 μg of PKC-δ siRNA premixed with the transfectant in a ratio of 1:8 provided optimal gene silencing effect without significant toxicity to the cells. B, inhibition of PKC-δ with siRNA resulted in an increase in Smad7 mRNA levels by real-time RT-PCR. The left panel is a representative histogram of real-time RT-PCR. Red line, solvent only; brown line, TGF-β (10 ng/ml); blue line, TGF-β (10 ng/ml) + PKC-δ siRNA. A left shift of threshold cycle from the red line (solvent only) to the brown line (TGF-β) in NF indicated an increase in Smad7 mRNA, while no significant change of threshold cycle was seen in PFF when exposed to TGF-β. β-Actin was used as controls showing no significant differences. Inhibition of PKC-δ with siRNA did not induce higher levels of Smad7 mRNA in NF. When PFF were incubated in TGF-β plus PKC-δ siRNA, the threshold cycle changed from the brown line (TGF-β only) to the blue line (TGF-β + PKC-δ siRNA) in PFF, indicating that inhibition of PKC-δ appeared to restore the capacity of TGF-β to induce Smad7 in PFF. The right panel shows relative values of Smad7 mRNA levels in bar graph form in NF (n = 2) and PFF (n = 3). *, p < 0.05; **, p < 0.01. C, inhibition of PKC-δ led to significant reduction in collagen synthesis. Parallel studies were conducted in PFF to determine collagen levels. Again, cells were cultured in medium alone, in medium containing TGF-β (10 ng/ml), or in combination of TGF-β plus PKC-δ siRNA for 24 h. The collagen content was determined using the Sircol collagen assay kit (Accurate). **, p < 0.01; ***, p < 0.001.
mRNA levels and, in parallel, reduced TGF-β-mediated collagen synthesis in PFF. The results suggest that the inhibition of PKC-δ may at least partially restore the negative feedback of TGF-β in PFF by facilitating an increase in Smad7.

IL-7 is a cytokine within the IL-2 receptor common γ chain (IL-2Rγc) superfamily (18). IL-7 receptor (IL-7Ra/γc) is comprised of a unique α chain (IL-7Rα) and a common γ (IL-7Rγ) subunit shared by other cytokine receptors including IL-2, IL-4, IL-9, and IL-15 (19). Activation of the JAK/STAT pathway has been implicated in IL-7 signaling (5, 20). Specifically, IL-7 is known to activate JAK1 through the ligand-specific IL-7Rα chain and subsequently activate STATs, while the shared IL-7Rγc subunit cross-linking by ligands activates JAK3, an upstream activator of a variety of signal transduction pathways (21). In vitro, IL-7 has the capacity to inhibit fibroblast TGF-β signaling in a JAK1/STAT1-dependent manner. In vivo, IL-7 decreases bleomycin-induced pulmonary fibrosis. Here we show for the first time that the counter-regulatory roles mediated by IL-7 and TGF-β in pulmonary fibroblast Smad7 expression and collagen synthesis are PKC-δ-dependent. PKC-δ may play a critical role in the pathogenesis of pulmonary fibrosis and serve as a molecular target for therapeutic intervention. The development of pathogenesis-based therapy for pulmonary fibrosis offers an entirely new avenue for therapeutic intervention.

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