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Original article:

IL-10 Inhibits Transforming Growth Factor-β-Induction of Type I Collagen mRNA Expression via Both JNK and p38 Pathways in Human Lung Fibroblasts

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ABSTRACT

Transforming growth factor-β (TGF-β) is a key factor for understanding the pathogenesis of fibrotic disorders such as idiopathic pulmonary fibrosis (IPF). We have demonstrated that interleukin-10 (IL-10) suppresses TGF-β-induced expression of type I collagen (COL1) mRNA in a human lung fibroblast cell line (WI-38). However, the inhibitory mechanism has not yet been clearly elucidated. Thus, in the current study, we investigate the effects of IL-10 blockade of TGF-β signaling which regulates COL1 mRNA expression. In WI-38 cells, IL-10 inhibits TGF-β-mediated phosphorylation of both, c-Jun HN_2-terminal kinase (JNK) and p38, but does not suppress TGF-β-mediated phosphorylation of Smad2 or affect TGF-β-upregulation of Smad7 mRNA expression. In addition, SP600125 and SB203580, specific inhibitors of JNK and p38, respectively, attenuate TGF-β-induced COL1 mRNA expression in WI-38 cells. These results suggest that IL-10 inhibits TGF-β-induced COL1 mRNA expression via both JNK and p38 pathways but not Smad pathways in WI-38 cells. This inhibitory mechanism may provide a novel insight into therapeutic strategies for fibrotic disorders such as IPF.

Keywords: IL-10, TGF-β, JNK, Smad, collagen

INTRODUTION

Idiopathic pulmonary fibrosis (IPF) is a devastating disease characterized by poor prognosis with a median survival of only two to three years (Bjoraker et al., 1998; Douglas et al., 2000; Schwartz et al., 1994) and by an unknown etiology. Transforming growth factor-β (TGF-β) is a key factor for understanding the pathogenesis of IPF. Immunohistochemical staining of TGF-β in lung sections of patients with IPF demonstrates a marked and consistent increase in TGF-β production in epithelial cells and macrophages compared to patients with nonspecific inflammation and to those with no inflammation or fibrosis (Khalil et al., 1991). In addition, we (Yoshida et al., 1995) and others (Sime et al., 1997) have found that overexpression of TGF-β in rat lungs results

in pulmonary fibrosis characterized by excessive accumulation of extracellular matrix (ECM) and limited inflammation. Type I collagen (COL1) is a major structural component of ECM and is synthesized by various cells including fibroblasts. TGF-β regulates COL1 gene expression in fibroblasts (Ignotz et al., 1987). However, the role of TGF-β signaling in COL1 gene expression in fibroblasts is not clearly understood.

TGF-β signaling is initiated following the binding of TGF-β to its membrane-bound receptors, which are classified as type I and type II receptors. TGF-β binding to the type II receptor recruits the type I receptor into a complex that results in phosphorylation of the transcription factors, Smad2 or Smad3. The phosphorylation of either Smad2 or Smad3 causes it to associate with Smad4. The complex translocates into the nucleus, where it regulates transcriptional responses together with additional DNA binding cofactors (Derynck et al., 1998). On the other hand, Smad7 is an intracellular antagonist for TGF-β signaling. Smad7 interferes directly with TGF-β-mediated activation of Smad2 by preventing its phosphorylation, association with Smad4, and nuclear accumulation (Hayashi et al., 1997; Nakao et al., 1997).

In addition to classical Smad pathways, mitogen-activated protein kinase (MAPK) pathways function downstream of TGF-β signaling (Engel et al., 1999; Hanafusa et al., 1999; Hu et al., 1999). The three major MAPK pathways have been characterized: extracellular signal-regulated kinase (ERK), c-Jun HN_2-terminal kinase (JNK) and p38. ERK plays a major role in cell proliferation (Portnoy et al., 2004) and differentiation (Kim et al., 1997) as well as in survival (Ahmad et al., 2004) mediated by various growth factors. On the other hand, JNK and p38 are activated by environmental stressors such as osmotic shock (Kultz et al., 1997), ultraviolet (UV) irradiation (Seo et al., 2002) and by pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α) (Goukassian et al., 2003) and interleukin-1 (IL-1) (Li et al., 2003), and play a critical role in apoptosis (Ichijo et al., 1997) and cytokine induction (Masuda et al., 2002).

Interleukin-10 (IL-10), first recognized for its ability to inhibit the activation and effector function of T cells, monocytes, and macrophages, is a multifunctional cytokine with diverse effects on most hematopoietic cell types. The principal function of IL-10 is to limit and ultimately terminate inflammatory responses (Moore et al., 2001). In addition to these activities, IL-10 modulates COL1 gene expression in skin fibroblasts (Reitamo et al., 1994; Wangoo et al., 1997). We have focused our attention on this effect of IL-10 and demonstrated that IL-10 suppresses TGF-β-induced COL1 mRNA expression in a human lung fibroblast cell line (WI-38) (Arai et al., 2000). However, the inhibitory mechanism of IL-10 on TGF-β-induced COL1 mRNA expression has not yet been clearly elucidated. This inhibitory mechanism may provide a novel insight into therapeutic strategies for fibrotic disorders such as IPF. Thus, in the current study, we investigate IL-10 blockade of TGF-β signaling which regulates COL1 mRNA expression in WI-38 cells.

MATERIALS AND METHODS

Cell culture

WI-38, a human lung fibroblast cell line, purchased from Health Science Research Resources Bank (Osaka, Japan) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 10 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

RNA preparation and Reverse transcription (RT)

WI-38 cells were seeded in DMEM containing 10% FBS at a density of 4×10^5 cells on a 60 mm-dish. Before confluence

was reached, the cells were starved for 24 h, and then treated with 10 ng/ml human TGF-β_1 (Sigma, St. Louis, Mo). In some experiments, the cells were preincubated with recombinant human IL-10 (Wako, Osaka, Japan), 10 μM SB203580 or 10 μM SP600125 (Calbiochem, San Diego, CA) for one hour before the administration of TGF-β_1. Total RNA from the cells was isolated with Isogen® (Nipponegene, Tokyo, Japan) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed with 25 μg/ml Oligo(dT)$_{12\text{-}18}$ (Invitrogen, Carlsbad, CA), 1× First strand Buffer (Invitrogen), 10 mM dithiothreitol, 0.5 mM deoxynucleotide triphosphates (dNTPs) and 5 U/ml SuperScript™ III (Invitrogen).

Polymerase chain reaction (PCR)

The cDNA mixture was subject to PCR amplification with following specific primers: sense 5'-CTGGTCCCAAGGGTAACAG-3' and antisense 5'-GCCAGGAGAACCACGTTC-3' for human COL1, sense 5'-TGCCTCCTGCACCACCAACTGC-3' and antisense 5'-AATGCCAGCCCCAGCGTCAAAG-3' for human glyceraldehyde-3-phospate dehydrogenase (GAPDH), and sense 5'-AAAGTGTTCCCTGGTTTCTCCATCAA GGC-3' and antisense 5'-CTACCGGCTGTTGAAGATGACCTCC AGCCAGCAC for human Smad7. The PCR cycles and the annealing temperature for each primer were as follows: 32 cycles at 58°C for COL1, 30 cycles at 65°C for Smad7, and 25 cycles at 60°C for GAPDH. After amplification, the PCR products were separated by electrophoresis on 2% agarose gels containing ethidium bromide. The density of the bands was quantified with FLUOR CHEM™ (Alpha Innotech, San Leandro, CA). The relative expression of COL1 and Smad7 mRNAs was quantified by assessing the ratio of the density of the COL1 and Smad7 bands, respectively, to GAPDH bands.

Protein extraction and Western blot analysis

WI-38 cells were seeded in DMEM containing 10% FBS at a density of 4×10^5 cells on a 60 mm-dish. Before confluence was reached, the cells were starved for 24 h, then treated with 10 ng/ml TGF-β_1 at various times in the presence or absence of 20 ng/ml IL-10. The cells were lysed with 100 μl RIPA buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonident P-40, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 1 mM phenylmethylsulfonylfluoride, 0.04 TIU/μl aprotinin, 1 mM sodium orthovanadate and 50 mM sodium fluoride. Protein concentrations were measured with the Detergent-Compatible (DC) protein assay (Bio-Rad, Hercules, CA). Samples were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated with rabbit primary antibodies against phospho-JNK, phospho-p38, phospho-ERK and phospho-Smad2 (all from Cell Signaling, Beverly, MA) at a 1:200 dilution for 1 h at room temperature, followed by incubation for another 1 h at room temperature with donkey anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Amersham, Piscataway, NJ) at a 1:2,000 dilution. The proteins were visualized after incubation of the blots using an enhanced chemiluminescence (ECL) system (Amersham). To determine the total amounts of JNK, p38, ERK, and Smad2, the blots were stripped and reprobed using antibodies against JNK, p38, ERK (Santa Cruz, Santa Cruz, CA), and Smad2 (Zymed, South San Francisco, CA) at a 1:1,000 dilution.

Statistical analysis

Data were expressed as means ± SD. Comparisons among groups were by ANOVA with Bonferroni/Dunn's tests used for post hoc analyses. Significance was accepted $p < 0.01$.

RESULTS

IL-10 inhibits TGF-β-induced COL1 mRNA expression in WI-38 cells.

We previously reported that TGF-β enhanced COL1 mRNA expression, and that IL-10 suppressed TGF-β-induced COL1 mRNA expression in a dose-dependent manner (Arai et al., 2000). To confirm that IL-10 inhibits TGF-β-induced COL1 mRNA expression, WI-38 cells were treated with TGF-β in the presence or absence of IL-10 for 24 h. The amounts of COL1 mRNA were determined by RT-PCR. As expected, TGF-β treatment increased expression of COL1 mRNA (Fig. 1). IL-10 decreased TGF-β-induced COL1 mRNA expression.

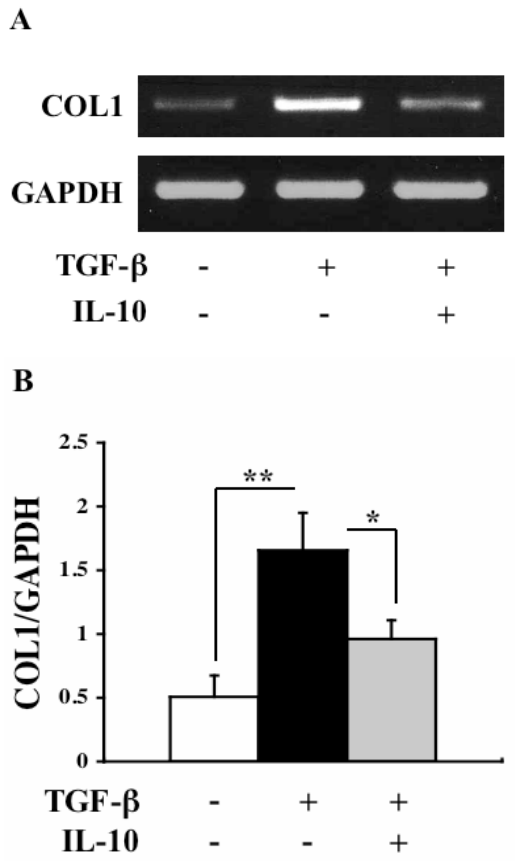

Figure 1: Effects of IL-10 on TGF-β-induced COL1 mRNA expression. WI-38 cells were treated with 10 ng/ml TGF-β in the presence or absence of 20 ng/ml IL-10 for 24 h. The amounts of COL1 mRNA were evaluated by RT-PCR, and expression was quantified by assessing the ratio of the density of COL1 bands to GAPDH bands. (A) Representative COL1 and GAPDH bands treated with TGF-β in the presence or absence of IL-10. (B) Ratio of the density of COL1 bands to GAPDH bands. Values represent means ± SD of 3 independent experiments. ** p <0.001 control vehicle vs. TGF-β.

IL-10 does not inhibit TGF-β-mediated phosphorylation of Smad2 in WI-38 cells

TGF-β signaling through Smad2 is initiated when the type I receptor kinase, activated by receptor II-mediated transphosphorylation, directly phosphorylates Smad2 at Ser465/467 (Macias-Silva et al., 1996). Many of the effects of TGF-β are mediated through Smad pathways. To determine Smad2 phosphorylation by TGF-β, WI-38 cells were treated with TGF-β at various times, and Western blot analysis was carried out using anti-phospho-Smad2 antibodies. As shown in Fig. 2A, Smad2 phosphorylation was increased at 15 min, reached a maximum at 30 min and remained elevated for up to 120 min after TGF-β treatment.

To determine whether IL-10 could block TGF-β-mediated Smad2 phosphorylation, WI-38 cells were preincubated with IL-10 before TGF-β treatment As shown in Fig. 2B, IL-10 did not decrease the amounts of TGF-β-mediated Smad2 serine phosphorylation.

IL-10 does not affect TGF-β-upregulation of Smad7 mRNA expression in WI-38 cells

Smad7 is an intracellular antagonist for TGF-β signaling. Smad7 functions by associating with the activated TGF-β type I receptor to block access to and phosphorylation of Smad2 (Hayashi et al., 1997; Nakao et al., 1997). To investigate whether IL-10 could affect the TGF-β-mediated Smad7 pathway, WI-38 cells were treated with TGF-β in the presence or absence of IL-10 for 24 h. The amounts of Smad7 mRNA were determined by RT-PCR. As shown in Fig. 3, TGF-β increased expression of Smad7 mRNA. However,

IL-10 did not affect TGF-β-upregulation of Smad7 mRNA expression. This result together with the result of Fig. 2 indicates that IL-10 does not affect TGF-β-mediated Smad pathways.

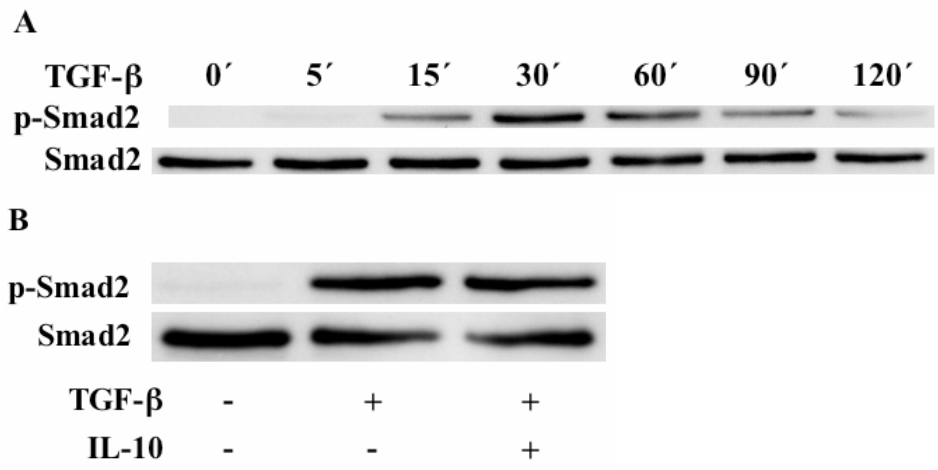

Figure 2: Effects of IL-10 on TGF-β-mediated phosphorylation of Smad2. WI-38 cells were treated with 10 ng/ml TGF-β at various times in the presence or absence of 20 ng/ml IL-10. The amounts of phospho-Smad2 were evaluated by Western blotting. (A) Time course of phosphorylated Smad2 induced by TGF-β. (B) Effects of IL-10 on TGF-β-induced phosphorylation of Smad2.

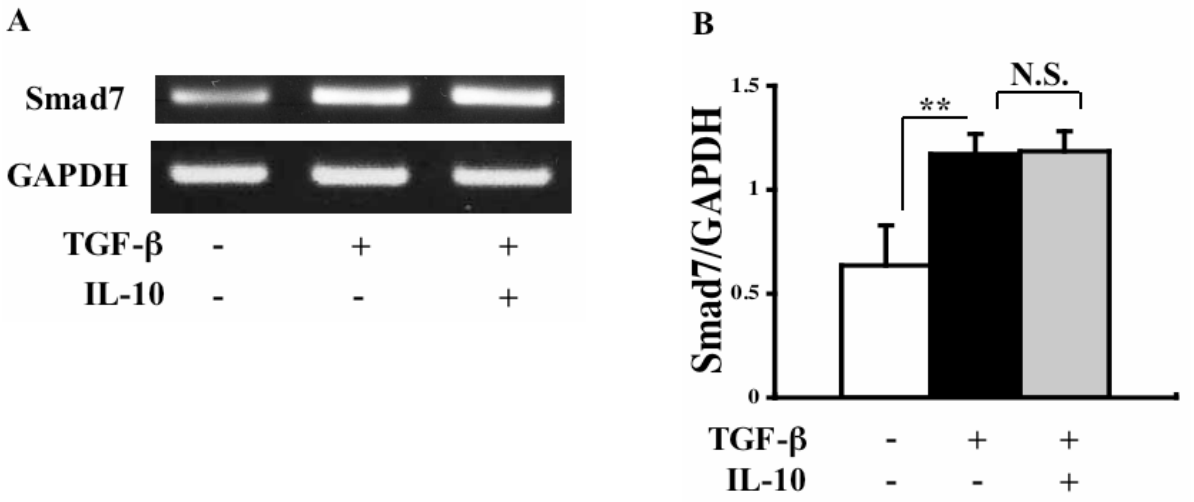

Figure 3: Effects of IL-10 on TGF-β-mediated mRNA expression of Smad7. WI-38 cells were treated with 10 ng/ml TGF-β in the presence or absence of 20 ng/ml IL-10 for 24 h. Smad7 mRNA was evaluated by RT-PCR, and expression was quantified by assessing the ratio of the density of Smad7 bands to GAPDH bands. (A) Representative Smad7 and GAPDH mRNA expression. (B) Densitometric analysis of Smad7 mRNA expression. Values represent means ± SD of 3 independent experiments. ** p <0.001 control vehicle vs. TGF-β. N.S. TGF-β vs. TGF-β plus IL-10. N.S. stands for Not Significant.

TGF-β results in phosphorylation of JNK and p38, but not ERK in WI-38 cells.

In addition to classical Smad pathways, it has been demonstrated in various cell types that TGF-β is capable of activating each of the MAPK pathways (Engel et al., 1999; Hanafusa et al., 1999; Hu et al., 1999). To determine whether TGF-β could activate

JNK, p38 and ERK, WI-38 cells were treated with TGF-β at various times, and Western blot analysis was carried out using anti-phospho-JNK, anti-phospho-p38, and anti-phospho-ERK antibodies, respectively. As shown in Fig. 4A, the amounts of phosphorylated JNK increased at 5 min, reached a maximum at 15 min and remained elevated for up to 120 min after TGF-β treatment. As shown in Fig. 4B, the amounts of phosphorylated p38 increased at 30 min, reached a maximum at 60 min and remained elevated for up to 120 min after TGF-β treatment. However, TGF-β did not phosphorylate ERK threonine/tyrosine residues (Fig. 4C).

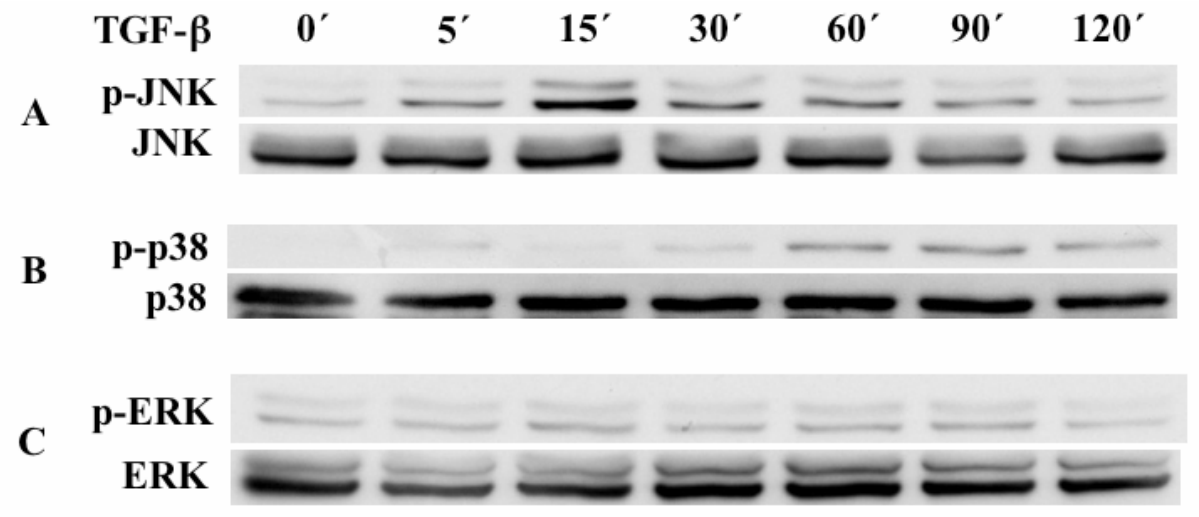

Figure 4: Effects of TGF-β on JNK, p38 and ERK. WI-38 cells were treated with 10 ng/ml TGF-β at various times. Phosphorylation of JNK, p38, and ERK was assessed by Western blotting using specific antibodies against phospho-JNK, phospho-p38 and phospho-ERK. (A) Amounts of phosphorylated JNK (46 kDa) (B) Amounts of phosphorylated p38 (C) Amounts of phosphorylated ERK1 (upper band, 44 kDa) and ERK2 (lower band, 42 kDa).

IL-10 inhibits TGF-β-mediated phosphorylation of both JNK and p38 in WI-38 cells

To determine whether IL-10 could block the TGF-β-mediated the JNK pathway or/and the p38 pathway, WI-38 cells were treated with TGF-β after a 60 min pre-incubation with IL-10. Western blot analysis was carried out using anti-phospho-JNK and anti-JNK antibodies as well as anti-phospho-p38 and anti-p38 antibodies. As shown in Fig. 5A, IL-10 decreased the amounts of TGF-β-induced phosphorylation of JNK. In addition IL-10 also reduced the amounts of TGF-β-mediated phosphorylation of p38 (Fig. 5B).

Specific inhibitors of JNK and p38, SP600125 and SB203580, respectively, attenuate TGF-β-induced COL1 mRNA expression in WI-38 cells

Finally, to determine whether the JNK pathway and/or the p38 pathway could regulate TGF-β-induced COL1 mRNA expression, WI-38 cells were treated with TGF-β in the presence or absence of 10 μM SP600125, a specific inhibitor of JNK (Han et al., 2001) or 10 μM SB203580, a specific inhibitor of p38 (Lee et al., 1994) for 24 h. The amounts of COL1 mRNA were determined by RT-PCR. As shown in Fig. 6A and B, SP600125 inhibited TGF-β-induced COL1 mRNA expression. In addition, SB203580 also suppressed TGF-β-induced COL1 mRNA expression (Fig. 6C and D).

These results indicate that both JNK and p38 pathways are involved in TGF-β-induced COL1 mRNA expression, suggesting that IL-10 inhibits TGF-β-induced COL1 mRNA expression via both JNK and p38 pathways.

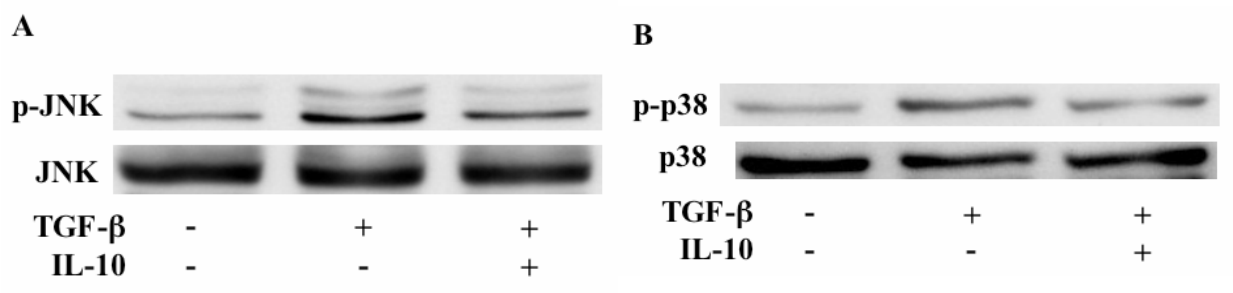

Figure 5: Effects of IL-10 on TGF-β-mediated phosphorylation of JNK and p38. WI-38 cells were treated with 10 ng/ml TGF-β following a 60 min pre-incubation of 20 ng/ml IL-10. Western blots were performed using phospho-JNK and JNK antibodies as well as phospho-p38 and p38 antibodies. (A) Effect of IL-10 on TGF-β-mediated phosphorylation of JNK (46 kDa). (B) Effect of IL-10 on TGF-β-mediated phosphorylation of p38.

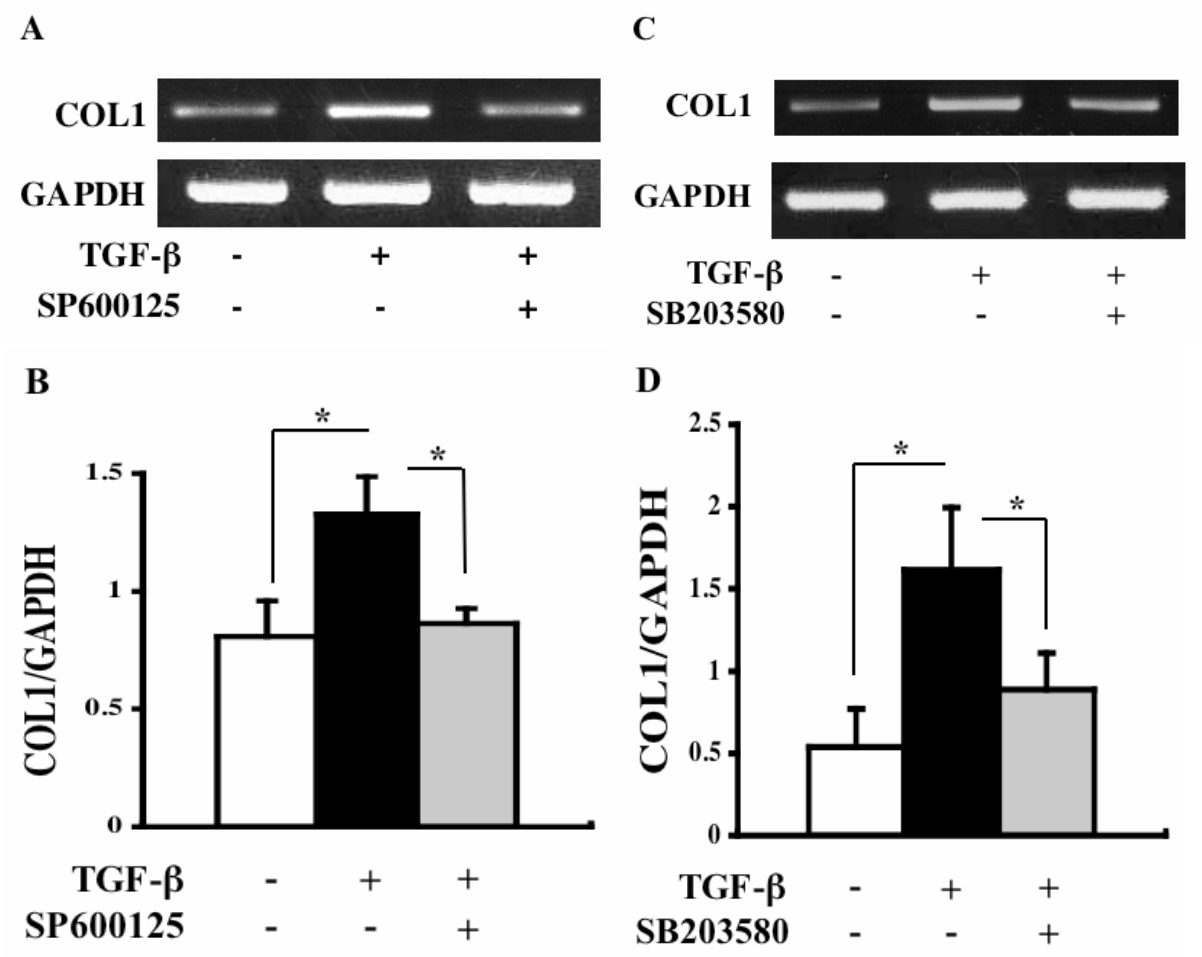

Figure 6: Effects of JNK and p38 inhibitors on TGF-β-induced COL1 mRNA expression. WI-38 cells were treated with 10 ng/ml TGF-β in the presence or absence of 10 μM SP600125 as a JNK inhibitor or 10 μM SB203580 as a p38 inhibitor for 24 h.

The amounts of COL1 mRNA were evaluated by RT-PCR and expression was quantified by assessing the ratio of the density of COL1 bands to GAPDH bands. Values represent means ± SD of 3 independent experiments. (A) Representative COL1 and GAPDH bands treated with control vehicle, TGF-β or TGF-β plus SP600125. (B) Ratio of density of COL1 bands to that of GAPDH bands. * p <0.01 control vehicle vs. TGF-β, TGF-β vs. TGF-β plus SP600125. (C) Representative COL1 and GAPDH bands treated with control vehicle, TGF-β or TGF-β plus SB203580. (D) Ratio of density of COL1 bands to that of GAPDH bands. * p <0.01 control vehicle vs. TGF-β, TGF-β vs. TGF-β plus SB203580.

DISCUSSION

There is accumulating evidence that IL-10 reduces TGF-β-induced COL1 gene expression in fibroblasts (Arai et al., 2000; Yamamoto et al., 2001). However, the IL-10 inhibitory mechanism of TGF-β-induced COL1 gene expression in fibroblasts has not yet been clearly established. Elucidation of this inhibitory mechanism may provide novel insight into therapeutic strategies for fibrotic disorders such as IPF. Thus, in the current study we investigate IL-10 blockade of TGF-β signaling which regulates COL1 mRNA expression. In WI-38 cells, IL-10 inhibits TGF-β-mediated phosphorylation of both JNK and p38, but does not suppress TGF-β-mediated phosphorylation of Smad2 or affect TGF-β-upregulation of Smad7 mRNA expression. In addition, specific inhibitors of JNK and p38 decrease TGF-β-induced COL1 mRNA expression. These results indicate that IL-10 inhibits TGF-β-induced COL1 mRNA expression via both JNK and p38 pathways but not Smad pathways.

Smad pathways have been reported to mediate TGF-β-induced COL1 gene expression, although the role of TGF-β signaling in COL1 gene expression is not clearly understood. Overexpression of Smad7 in renal tubular epithelial cells reduces COL1 mRNA expression with a marked inhibition of TGF-β-mediated Smad2 activation (Li et al., 2002). Gene transfer of Smad7 prevents bleomycin-induced lung fibrosis (Nakao et al., 1999), suggesting that Smad7 may have applicability in the treatment of pulmonary fibrosis. In WI-38 cells, IL-10 does not suppress TGF-β-induced phosphorylation of Smad2 or affect TGF-β upregulation of Smad7 mRNA expression. In this study, we demonstrate that IL-10 does not affect TGF-β-Smad pathways, indicating that the other mechanisms are involved in the inhibitory effect of IL-10 on COL1 gene expression.

In addition to classical Smad pathways, TGF-β is capable of activating each of the three MAPK pathways in various cell types including lung fibroblasts. TGF-β results in phosphorylation of JNK and p38, but not ERK in a human lung fibroblast cell line (HLF-1) (Utsugi et al., 2003) as well as in phosphorylation of JNK, p38 and ERK in another human lung fibroblast cell line (HLF) (Hashimoto et al., 2001). In WI-38 cells, TGF-β activates JNK and p38, but not ERK. TGF-β-activated kinase 1 (TAK1) was first identified as one of the MAPK kinase kinase family (Yamaguchi et al., 1995) and appears preferably to be an upstream regulator of JNK (Wang et al., 1997) and p38 (Hanafusa et al., 1999) pathways in TGF-β signaling. In TGF-β-stimulated WI-38 cells, it is possible that TAK1 regulates JNK and p38 phosphorylation and activation.

IL-10 has been found to inhibit MAPK pathways activated by several stimuli in different cell types. IL-10 suppresses LPS-induced p38 phosphorylation in macrophages (Kontoyiannis et al., 2001) and IL-1β-stimulated JNK activation in brain tissue (Kelly et al., 2001). In TGF-β-treated WI-38 cells, IL-10 inhibits both JNK and p38 phosphorylation. The effects of IL-10 are mediated by the IL-10 receptor on the target

cells including human fibroblasts (Wang et al., 1999). Activation of the IL-10 receptor has been found to activate the JAK-STAT pathway (Finbloom et al., 1995). The detailed mechanism by which IL-10 targets TGF-β-mediated both the JNK and p38 pathways has not been investigated in the current study. IL-10 signaling could directly interfere with TGF-β-mediated JNK and p38 pathways. However, further studies are needed to clarify how IL-10 interferes with TGF-β-mediated JNK and p38 pathways.

MAPK has been also demonstrated to modulate COL1 gene expression by several stimuli in mammalian cells, although the role of TGF-β signaling in COL1 gene expression is not clearly understood. JNK modulates UV-induced gene expression of COL1 in rat hepatic stellate cells (Chen et al., 1999). Moreover, p38 regulates both TNFα-induced and TGF-β-induced COL1 gene expression in the same cell line (Varela-Rey et al., 2002). In our study, inhibition of either JNK or p38 reduces TGF-β-induced COL1 mRNA expression. Our findings indicate that both JNK and p38 are important for modulating TGF-β-induced COL1 mRNA expression. This concept is supported by recent evidence that a dominant negative TAK1, which appears to be an upstream regulator of JNK (Wang et al., 1997) and p38 (Hanafusa et al., 1999) pathways in TGF-β signaling, inhibits cellular fibrotic responses induced by TGF-β (Ono et al., 2003). Given that no exogenous Smad inhibitor exists to date, one of the new insights that our study has provided is the clarification of the inhibitory mechanism of IL-10. JNK and p38 inhibitors, which already do exist, may represent a more feasible therapeutic strategy for fibrotic disorders such as IPF.

FR167653 was first discovered as a cytokine suppressive agent *in vivo* (Yamamoto et al., 1996), which was later found to resemble the chemical structure of SB203580 to belong to one of p38 inhibitors (Takahashi et al., 2001). Our previous investigations demonstrated that both IL-10 (Arai et al., 2000) and FR167653 (Matsuoka et al., 2002) suppress bleomycin-induced pulmonary fibrosis in a murine model. These similar *in vivo* findings can be explained by our new *in vitro* evidence that both IL-10 and SB203580 reduce TGF-β-induced COL1 mRNA expression, because in this model bleomycin has been demonstrated to induce a TGF-β-dependent induction of pulmonary fibrosis with increased collagen and hydroxyproline synthesis (Kolb et al., 2001).

In conclusion, IL-10 suppresses TGF-β-induced COL1 mRNA expression via both JNK and p38 pathways but not Smad pathways in WI-38 cells. This inhibitory mechanism may provide a new insight into therapeutic strategies for fibrotic disorders such as IPF.

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