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Interleukin-4 Regulates Connective Tissue Growth Factor Expression in Human Lung Fibroblasts

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Abstract Transforming growth factor-β (TGF-β) and interleukin-4 (IL-4) have fibrogenic properties and induce extracellular matrix production in a variety of lung diseases. Connective tissue growth factor (CTGF) is a matrix signaling molecule stimulated by TGF-β that in part mediates α1(I) collagen mRNA expression. In these studies, the regulation of CTGF expression by IL-4 in human lung fibroblasts was examined. Following 6 h of stimulation with IL-4, basal CTGF mRNA levels were unchanged as assessed by Northern blot analysis. However, IL-4 attenuated the TGF-β-stimulated induction of CTGF mRNA expression by 50%. This effect was selective because IL-4 did not affect fibronectin or α1(I) collagen mRNA expression induced by TGF-β. Experiments employing the transcriptional inhibitor actinomycin D suggest that IL-4 did not affect the stability of the CTGF mRNA. Transient transfection assays with 3TP-Lux, a luciferase gene controlled by a TGF-β inducible promoter, and with a CTGF promoter construct indicate that IL-4 interfered with the TGF-β-induced transcriptional activation of the CTGF gene. J. Cell. Biochem. 85: 496–504, 2002.

Key words: transforming growth factor-β; Smad; Stat; fibrosis

Connective tissue growth factor (CTGF), a 38-kD cysteine-rich protein, stimulates the production of extracellular matrix elements by fibroblasts [Stratton et al., 2001]. Increased CTGF levels are demonstrated in many fibrotic human tissues, including lung [Ziesche et al., 1999], skin [Igarashi et al., 1996], liver [Abou-Shady et al., 2000], kidney [Ito et al., 1998], and blood vessels [Oemar et al., 1997]. In addition, enhanced CTGF expression is identified in the bronchoalveolar lavage from patients with fibrotic lung disease [Allen et al., 1999]. In vitro, transforming growth factor-β (TGF-β) activates CTGF gene transcription in human lung fibroblasts [Ricupero et al., 1999].

Interactions between inflammatory cells and fibroblasts contribute to the pathogenesis of a variety of lung diseases that are characterized by excess collagen deposition, including idiopathic pulmonary fibrosis and asthma. TGF-β and interleukin-4 (IL-4) are effector substances with fibrogenic properties that stimulate extracellular matrix production in these diseases [Minshall et al., 1997; Doucet et al., 1998]. TGF-β signal transduction is mediated by Smad proteins [Massague, 1998; Massague and Chen, 2000; Massague and Wotton, 2000] and TGF-β induces α1(I) collagen mRNA by a mechanism that may be in part dependent on CTGF [Duncan et al., 1999; Ricupero et al., 1999]. IL-4 is a cytokine product of T lymphocytes, mast cells, eosinophils, and basophils and IL-4 receptors are expressed on fibroblasts [Doucet et al., 1998].

CTGF mRNA expression is upregulated in a murine model of pulmonary fibrosis induced by bleomycin [Lasky et al., 1998]. The susceptibility of mice to the induction of pulmonary fibrosis by bleomycin is strain dependent [Baecher-Allan and Barth, 1993; Lasky et al., 1998]. For example, C57BL/6 mice are sensitive to the effects of bleomycin, whereas BALB/c mice are resistant. Following treatment with bleomycin, IL-4 mRNA expression is similar in both strains of mice, however, IL-4 receptor mRNA is

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induced to a greater extent in the sensitive C57BL/6 mice [Baecher-Allan and Barth, 1993]. This suggests that the modulatory effects of IL-4 on fibrosis are due to the expression of both the ligand and the receptor.

In these studies, the regulation of CTGF mRNA expression by IL-4 in human lung fibroblasts was examined. IL-4 did not affect basal expression of CTGF mRNA, but IL-4 attenuated the transcriptional activation of the CTGF gene by TGF-β. Specifically, it appears that IL-4 interfered with TGF-β induction of the CTGF promoter.

MATERIALS AND METHODS

Tissue Culture

Human embryonic lung fibroblasts (IMR-90, Institute for Medical Research) were grown in Dulbecco's Modified Eagle's Medium supplemented with 0.37 g sodium bicarbonate/100 ml, 10% (v/v) fetal bovine serum (FBS), 100 U penicillin/ml, 10 μg streptomycin/ml, 0.1 mM pyruvate, and 0.1 mM non-essential amino acids. After confluence, the serum content of the medium was reduced to 0.4% FBS for 48 h. Cell numbers were determined using an electronic particle counter (Coulter Counter ZM).

Northern Blot Analysis

Total cellular RNA was isolated using RNAwiz (Ambion). RNA was quantified by absorbance at 260 nm. Purity was determined by absorbance at 280 and 310 nm. RNA (10 μg) was electrophoresed through a 1% agarose/6% formaldehyde gel, and transferred to a nylon membrane. RNA loading was assessed by ethidium bromide staining of ribosomal bands and by cohybridization with glyceraldehyde 3-phosphate dehydrogenase (GADPH). The membrane was exposed to x-ray film for autoradiography at several time points to ensure that the bands could be quantified by densitometry within the linear range. The human CTGF cDNA probe was generated by polymerase chain reaction using primers previously reported [Ricupero et al., 2000]. The α1(I) collagen cDNA probe was derived from a rat α1(I) collagen cDNA and specifically binds human α1(I) collagen mRNA [Genovese et al., 1984].

Western Blot Analysis

Samples were prepared from the cell layer of fibroblasts grown in 100-mm tissue culture dishes. The cell layer was dissolved in RIPA buffer at 4°C and centrifuged 14,000*g* for 10 min. The protein yield was determined by assay (Bio-Rad Laboratories). SDS–PAGE and Western blotting was performed using 7.5% polyacrylamide minigels as previously described [Kim et al., 1995]. Proteins were transferred to a nitrocellulose membrane and blocked for 1 h at room temperature with 10% evaporated milk in phosphate buffer saline with 0.1% Tween then incubated for 24 h at 4°C with a 1:1,000 dilution of rabbit anti-CTGF antibody (provided by D.R. Brigstock). Proteins were detected using a chemiluminescence kit (NEN), and the membrane was exposed to x-ray film for autoradiography. FibroGen provided recombinant human CTGF.

Transfection Assays

An 807-bp fragment of the human CTGF promoter is coupled to the luciferase reporter gene and cloned into the pGL2-Basic vector (Promega) (provided by G.R. Grotendorst) [Grotendorst et al., 1996]. The TGF-β-inducible luciferase reporter construct, 3TP-Lux, is cloned into the pGL2-Basic vector (Promega) [Ricupero et al., 2000]. Plasmids were transiently transfected into fibroblasts using Lipofectamine PLUS reagent (Life Technologies) and luciferase activity was measured by assay (Promega).

Materials

Recombinant human IL-4 and porcine platelet-derived TGF-β1 were obtained from R&D Systems. LY294002, apigenin, and PD98059 were obtained from Calbiochem. Actinomycin D, cycloheximide (CHX), and recombinant human insulin were obtained from Sigma.

Statistics

A Student's test was used for means of unequal size. Probability values less than 0.05 were considered significant.

RESULTS

The effects of IL-4 and TGF-β on CTGF mRNA and protein expression were examined in human lung fibroblasts (Fig. 1). Consistent with previous reports, TGF-β (1 ng/ml) induced CTGF mRNA and protein levels at 6 h. IL-4 (25 ng/ml) did not affect basal CTGF mRNA or protein levels. However, IL-4 attenuated the

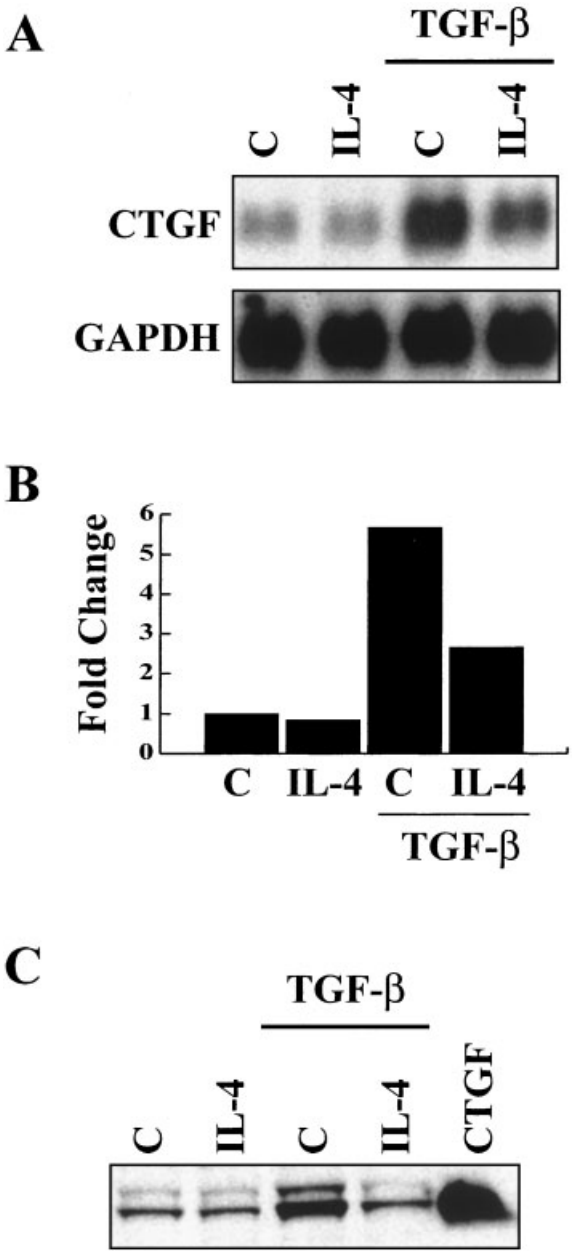


Fig. 1. Effect of IL-4 on TGF-β-stimulated induction of CTGF mRNA and protein expression in human lung fibroblasts. Confluent, quiescent fibroblast cultures were left untreated (C) or treated with IL-4 (25 ng/ml) and/or TGF-β (1 ng/ml) as indicated for 6 h. **A**: Northern blot analysis was performed. Total RNA was isolated and 10 μg per lane was resolved electrophoretically. CTGF was detected using a probe for CTGF mRNA and loading was assessed using a probe for GAPDH mRNA as described. Representative of three independent experiments. **B**: Densitometry analysis was performed. The histogram plots fold change in optical density above control of the representative Northern blot. **C**: Western blot analysis was performed. Total protein was harvested from the cell layer and 100 μg per lane was resolved by SDS–PAGE. Recombinant human CTGF (25 μg) was used to identify the molecular weight of CTGF. CTGF was detected with an anti-CTGF antibody as described.

TGF-β induction of CTGF mRNA and protein expression in human lung fibroblasts at 6 h in three independent experiments. Densitometry analysis of the representative Northern blot revealed that IL-4 decreased TGF-β-induced CTGF mRNA levels by 50%. Increasing the concentration of TGF-β (10 ng/ml) did not alter the attenuation of the TGF-β signal by IL-4 (data not shown).

A dose–response relation between IL-4 and CTGF mRNA was determined to confirm that IL-4 did not affect basal CTGF mRNA expression (Fig. 2A). IL-4 in concentrations ranging from 0.1 to 100 ng/ml did not alter CTGF mRNA levels at 6 h. A time-course study showed that IL-4 (25 ng/ml) decreased TGF-β-induced CTGF mRNA expression at 2 h with a more pronounced effect at 4 and 6 h (Fig. 2B). Because IL-4 and IL-13 share several biological properties and their receptors are expressed on fibroblasts [Doucet et al., 1998], the effect of IL-13 on

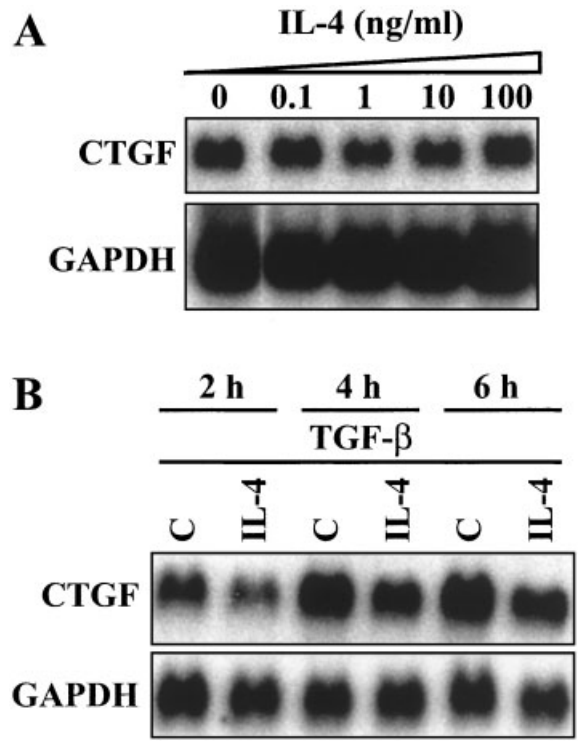


Fig. 2. **A**: Effect of IL-4 on basal CTGF mRNA expression in human lung fibroblasts. Confluent, quiescent fibroblast cultures were left untreated (C) or treated with varying concentrations of IL-4 as indicated for 4 h. Northern blot analysis was performed as described. **B**: Time-course for the effect of IL-4 on TGF-β-stimulated induction of CTGF mRNA expression in human lung fibroblasts. Confluent, quiescent fibroblast cultures were treated with TGF-β (1 ng/ml) with or without IL-4 (25 ng/ml) for varying times as indicated. Northern blot analysis was performed as described.

the TGF-β induction of CTGF mRNA levels was also examined and shown to be similar to that demonstrated with IL-4 (data not shown).

To determine whether IL-4 specifically attenuates the TGF-β induction of CTGF mRNA expression, Northern blot analyses were performed using probes for fibronectin and α1(I) collagen (Fig. 3). IL-4 did not affect TGF-β-induced fibronectin or α1(I) collagen mRNA levels at 6 or 24 h. Consistent with previous studies, IL-4 induced fibronectin and α1(I) collagen mRNA levels at 24 h [Postlethwaite et al., 1992].

TGF-β increases the steady-state level of CTGF mRNA by activating transcription of the gene [Ricupero et al., 1999]. The effect of IL-4 on TGF-β-induced CTGF gene transcription was assessed using the transcriptional inhibitor actinomycin D (Fig. 4A,B). Northern blot analysis revealed that following the administration of actinomycin D, the rate of decay of CTGF mRNA from fibroblasts treated with TGF-β and IL-4 was not reduced compared with the rate of decay observed in fibroblasts treated with TGF-β, indicating that IL-4 did not affect the stability of the CTGF mRNA. The effect of IL-4 on TGF-β-induced CTGF mRNA expression was further characterized using CHX to determine whether IL-4 activity required protein synthesis (Fig. 4C). CHX did not interfere with the IL-4 attenuation of TGF-β-induced CTGF mRNA levels at 6 h as assessed by Northern blot analysis.

The IL-4Rα chain of the IL-4 receptor contains a cytoplasmic region that is highly homologous to a region found in the insulin and insulin growth factor-1 receptors [Keegan et al., 1994]. This region is required for binding and phosphorylating insulin receptor substrate-1 (IRS-1). IRS-1 and phosphoinositol-3 kinase (PI3K) are activated in response to IL-4 [Wang et al., 1993; Zamorano et al., 1996]. To determine whether activation of IRS-1 was involved in the attenuation of TGF-β-induced CTGF mRNA expression, fibroblasts were left untreated or treated with insulin, TGF-β, or both insulin and TGF-β (Fig. 5A). Insulin did not decrease the induction of CTGF mRNA levels by TGF-β at 6 h. The role of PI3K was then examined using LY294002 to inhibit PI3K activation (Fig. 5B). LY294002 did not affect the attenuation of TGF-β-induced CTGF mRNA levels by IL-4 at 6 h.

In some systems, IL-4 activates the mitogen-activated protein kinase (MAPK) signaling pathway [Duronio et al., 1992; Welham et al., 1994]. To assess whether MAPK activation was involved in the IL-4 attenuation of TGF-β-induced CTGF mRNA expression in human lung fibroblasts, inhibitor studies were performed (Fig. 6). Fibroblasts were incubated with apigenin, an inhibitor of MAPK, or PD98059, an inhibitor of mitogen-activated protein kinase kinase (MEK), prior to treatment with IL-4 and TGF-β. These inhibitors did not affect the attenuation of TGF-β-induced CTGF mRNA expression by IL-4 at 6 h.

Transient transfection assays using 3TP-Lux, a TGF-β-inducible luciferase reporter construct, were used to evaluate the effect of

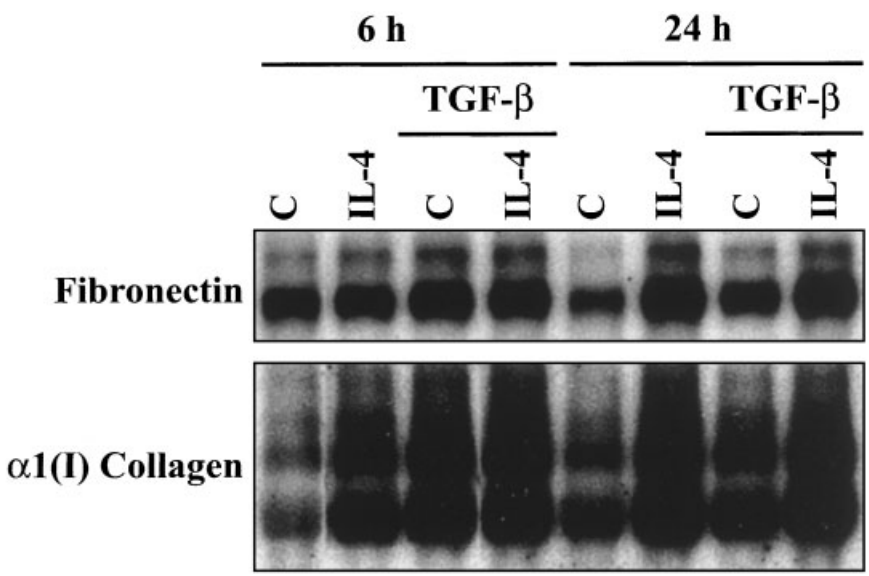


Fig. 3. Effect of IL-4 on TGF-β-stimulated induction of fibronectin and α1(I) collagen mRNA expression in human lung fibroblasts. Confluent, quiescent fibroblast cultures were left untreated (C) or treated with IL-4 (25 ng/ml) and/or TGF-β (1 ng/ml) for 6 or 24 h as indicated. Total RNA was isolated and 10 μg per lane was resolved electrophoretically. Northern blot analyses were performed using cDNA probes for fibronectin and α1(I) collagen mRNA.

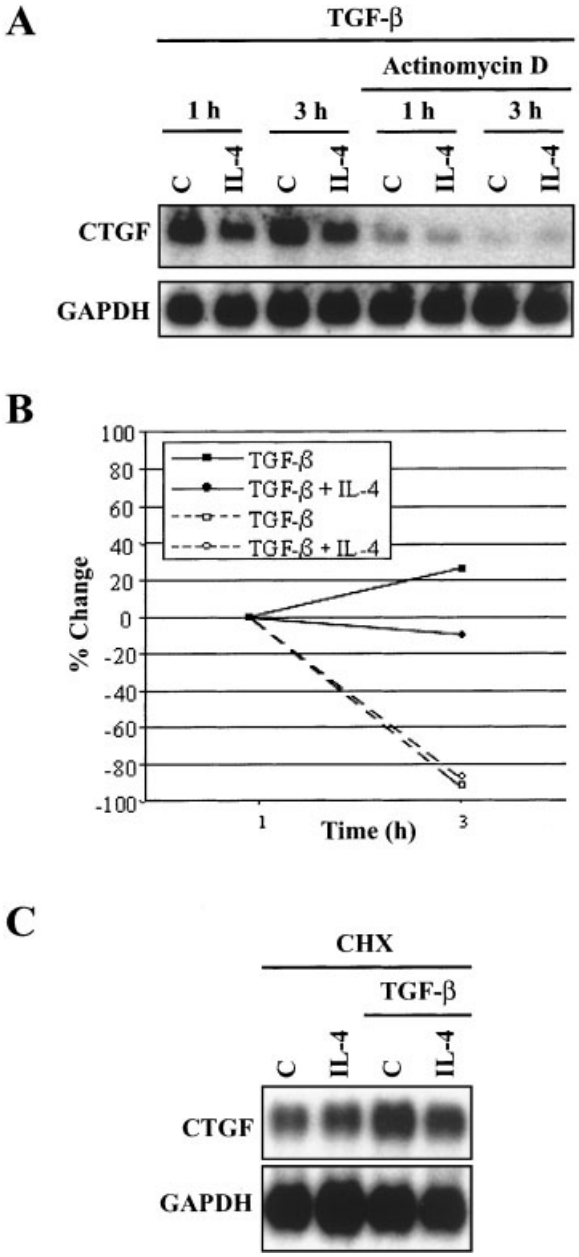


Fig. 4. **A**: Effect of actinomycin D on IL-4 attenuation of TGF-β-induced CTGF mRNA expression in human lung fibroblasts. Confluent, quiescent fibroblast cultures were treated with TGF-β (1 ng/ml) with or without IL-4 (25 ng/ml) for 2 h prior to the addition of actinomycin D (5 μM) to the indicated cultures. Cultures were then incubated for an additional 1 or 3 h as indicated. Northern blot analysis was performed as described. **B**: Densitometry analysis was performed. The line graph plots the percent change in optical density at each time point of the representative Northern blot. The open square and circle indicate the addition of actinomycin D. **C**: Effect of CHX on IL-4 attenuation of TGF-β-induced CTGF mRNA expression in human lung fibroblasts. Confluent, quiescent fibroblast cultures were incubated with CHX (5 μM) for 30 min and were then left untreated (C) or treated with IL-4 (25 ng/ml) and/or TGF-β (1 ng/ml) as indicated for 6 h. Northern blot analysis was performed as described. Representative of two independent experiments.

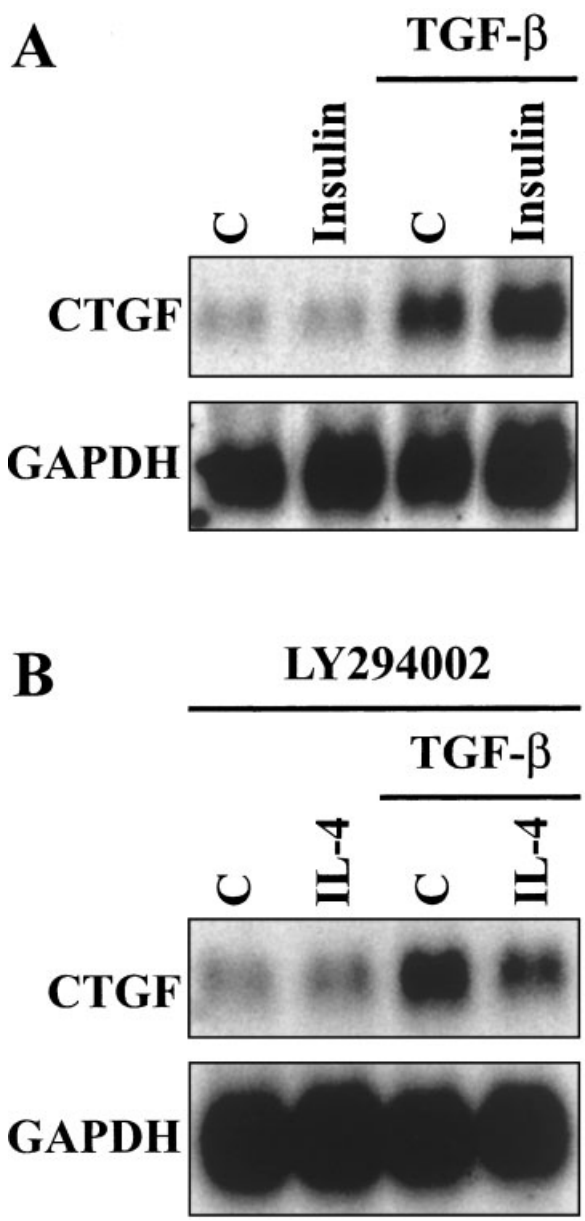
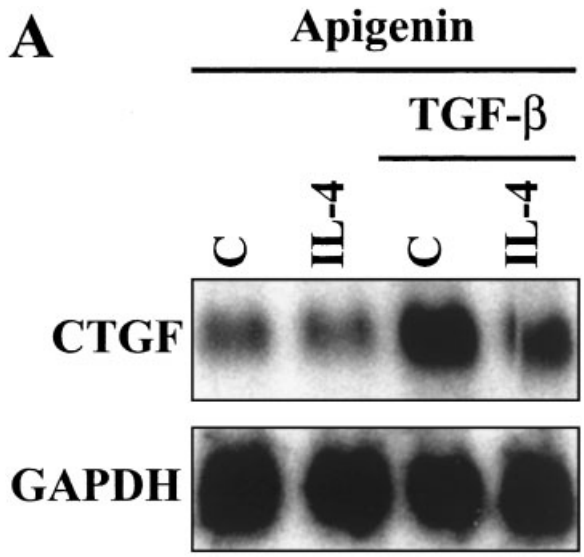


Fig. 5. **A**: Effect of insulin on TGF-β-stimulated induction of CTGF mRNA expression in human lung fibroblasts. Confluent, quiescent fibroblast cultures were left untreated (C) or treated with insulin (2 U/ml) and/or TGF-β (1 ng/ml) as indicated for 6 h. Northern blot analysis was performed as described. **B**: Effect of LY294002 on IL-4 attenuation of TGF-β-induced CTGF mRNA expression in human lung fibroblasts. Confluent, quiescent fibroblast cultures were incubated with LY294002 (25 μM) for 10 min, and were then left untreated (C) or treated with IL-4 (25 ng/ml) and/or TGF-β (1 ng/ml) as indicated for 6 h. Northern blot analysis was performed as described. Representative of two independent experiments.

IL-4 on TGF-β signal transduction (Fig. 7). Fibroblasts treated with TGF-β increased luciferase activity 12-fold, whereas those treated with IL-4 had no affect on luciferase activity.



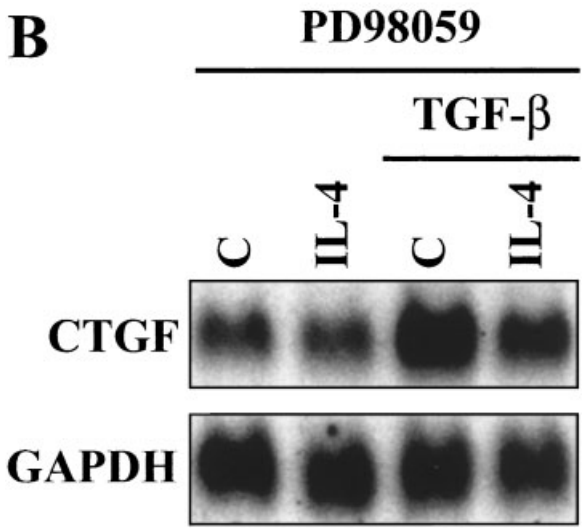


Fig. 6. Effect of (**A**) apigenin and (**B**) PD98059 on IL-4 attenuation of TGF-β-induced CTGF mRNA expression in human lung fibroblasts. Confluent, quiescent fibroblast cultures were incubated with apigenin (20 μM) for 10 min or PD98059 (20 μM) for 10 min, and were then left untreated (C) or treated with IL-4 (25 ng/ml) and/or TGF-β (1 ng/ml) as indicated for 6 h. Northern blot analyses were performed as described. Representative of two independent experiments.

Fibroblasts treated with both IL-4 and TGF-β increased luciferase activity 10-fold. Results of two independent experiments showed that there were no statistically significant differences in luciferase activity between untreated fibroblasts and IL-4-treated fibroblasts or between fibroblasts treated with TGF-β and fibroblasts treated with both IL-4 and TGF-β.

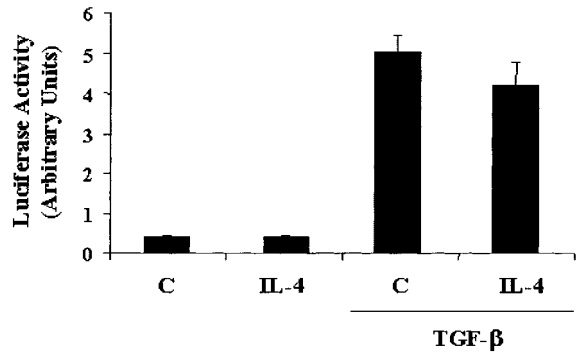


Fig. 7. Effect of IL-4 on TGF-β-induced 3TP-Lux activity in human lung fibroblasts. Fibroblasts were transiently transfected with the 3TP-Lux reporter construct as described. Fibroblasts were then left untreated (C) or treated with IL-4 (25 ng/ml) and/or TGF-β (1 ng/ml) as indicated for 6 h. Luciferase assay was performed as described. The histogram plots the luciferase activity (mean + standard deviation) of triplicate values and is representative of two independent experiments.

To determine whether IL-4 affects the transcriptional activation of the CTGF gene by TGF-β, a CTGF promoter construct was used in transient transfection assays (Fig. 8). Fibroblasts treated with IL-4 had no affect on luciferase activity. Fibroblasts treated with TGF-β increased luciferase activity greater than six-fold. Treatment with both IL-4 and TGF-β increased luciferase activity less than four-fold. IL-4 decreased the TGF-β induction of the CTGF promoter activity by 40%. Results of two independent experiments showed that

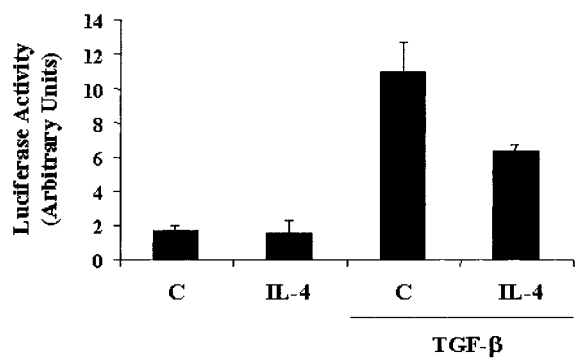


Fig. 8. Effect of IL-4 on TGF-β-induced CTGF promoter activity in human lung fibroblasts. Fibroblasts were transiently transfected with the CTGF promoter construct as described. Fibroblasts were then left untreated (C) or treated with IL-4 (25 ng/ml) and/or TGF-β (1 ng/ml) as indicated for 24 h. Luciferase assay was performed as described. The histogram plots the luciferase activity (mean + standard deviation) of triplicate values and is representative of two independent experiments.

there was a statistically significant difference in luciferase activity between fibroblasts treated with TGF-β and fibroblasts treated with both IL-4 and TGF-β ($P < 0.05$). There was no statistically significant difference in luciferase activity between untreated fibroblasts and IL-4-treated fibroblasts.

DISCUSSION

In these studies, the effect of IL-4 on CTGF expression in human lung fibroblasts was examined. IL-4 decreased the rate of transcription of CTGF mRNA induced by TGF-β as assessed by studies using actinomycin D. Moreover, IL-4 attenuated the CTGF promoter activity stimulated by TGF-β. IL-4 did not alter the basal level of CTGF mRNA suggesting that IL-4 did not directly affect CTGF gene transcription or mRNA stability. IL-4 appears to attenuate the TGF-β-induced increase in the steady-state level of CTGF mRNA by interfering with CTGF gene transcription rather than with CTGF mRNA stability.

TGF-β signal transduction is mediated by the Smad family of proteins [Massague, 1998; Massague and Chen, 2000; Massague and Wotton, 2000]. Following TGF-β stimulation, Smad2 and Smad3 are phosphorylated, and form heteromeric complexes with Smad4. These complexes translocate to the nucleus and regulate gene transcription by binding DNA directly or in association with other transcription factors. Transcriptional activation may be further regulated by coactivators or corepressors binding to the Smad complex. The experimental evidence presented did not directly show that IL-4 interfered with Smad-mediated signaling, however, indirect evidence was provided using the 3TP-Lux and CTGF promoter constructs. Although IL-4 did not affect basal or TGF-β-induced activity of 3TP-Lux, mediators of IL-4 signal transduction may alter the association of Smads and cofactors necessary for specific TGF-β-induced gene transcription. Smads bind the consensus sequence CAGAC with low affinity. The association of Smads with DNA-binding cofactors, such as AP-1 or FAST, increase their affinity and their regulatory specificity [Chen et al., 1997; Wong et al., 1999]. IL-4 may be antagonizing the association of Smad3/4 with specific cofactors necessary for TGF-β-stimulated transcriptional activation of the CTGF gene.

IL-4 may be interfering with Smad-mediated activation of CTGF gene transcription by two other less likely mechanisms. First, the 3TP-Lux reporter construct is responsive to Smad2/4 to a lesser degree than to Smad3/4 [Yingling et al., 1997; Xu et al., 2000; Piek et al., 2001]. Smad2 and Smad3 may be differentially activated by mediators of IL-4 signaling. Consistent with this possibility, calmodulin increases Smad1 activity, while decreasing Smad2 activity in Xenopus embryos [Scherer and Graff, 2000]. Second, IL-4 may activate a corepressor or inactivate a coactivator of Smad-mediated gene transcription of CTGF. For example, the corepressor Ski can suppress CTGF promoter activity induced by TGF-β [Holmes et al., 2001], although it is unlikely that IL-4 is acting in this way because Ski also suppresses TGF-β-induced 3TP-Lux activity [Xu et al., 2000].

Fibrotic skin lesions in scleroderma are characterized by high basal expression of CTGF [Shi-wen et al., 2000]. In addition, increased CTGF promoter activity in scleroderma skin fibroblasts is independent of mutations to the Smad binding site [Holmes et al., 2001], suggesting that basal transcriptional activation of the CTGF gene is not Smad mediated. The lack of effect of IL-4 on the constitutive expression of CTGF mRNA, but its ability to interfere with TGF-β-induced expression, also suggest that the basal level of CTGF mRNA is not dependent on TGF-β activation in human lung fibroblasts.

The induction of Smad7 expression can antagonize TGF-β signaling. Interferon-γ (IFN-γ) activation of Stat1 increases Smad7 expression that subsequently inhibits Smad3 phosphorylation and signal transduction [Ulloa et al., 1999]. Although the induction of Smad7 by IFN-γ provides a potential for interaction between the Stat and Smad signaling pathways, it is unlikely that IL-4 is inducing Smad7 because the inhibition of protein synthesis by CHX did not interfere with the effect of IL-4 on TGF-β signaling. In addition, the induction of Smad7 expression would be expected to decrease the activity of 3TP-Lux. IL-4 did not affect the basal or the TGF-β-induced activity of 3TP-Lux. Finally, the increased expression of Smad7 would not be expected to result in gene specific transcriptional regulation. IL-4 attenuated the TGF-β induction of CTGF mRNA expression, but not fibronectin or α1(I) collagen mRNA expression.

IL-4 induces the phosphorylation of JAK proteins that activate Stat6. In addition, IRS-1 and PI3K are activated in response to IL-4 and insulin [Wang et al., 1993; Zamorano et al., 1996]. The use of insulin to activate IRS-1 and LY294002 to inhibit PI3K did not affect the ability of IL-4 to attenuate the TGF-β induction of CTGF mRNA levels, suggesting that IRS-1 and PI3K activation by IL-4 is not implicated in this effect. Although IL-4 also activates MAPK [Duronio et al., 1992], this pathway is not involved because inhibitors of MAPK and MEK did not interfere with the effect of IL-4 on TGF-β-induced CTGF mRNA levels.

IL-4 may participate in the initiation of fibrosis in the acute response to tissue inflammation or injury. During this initial response, IL-4 may act by attenuating some of the fibrogenic effects of TGF-β, specifically, the induction of CTGF. High basal levels of CTGF are associated with the maintenance of fibrosis and appear independent of TGF-β or IL-4 regulation. In a mouse model of skin fibrosis, the subcutaneous injection of TGF-β and CTGF together resulted in a persistent fibrotic response, whereas the injection of TGF-β or CTGF alone resulted in only transient fibrosis [Mori et al., 1999].

In summary, IL-4 appears to attenuate the transcriptional activation of the CTGF gene by TGF-β. The initiation and resolution of the fibrotic response to tissue inflammation or injury may be dependent on interactions between IL-4 and TGF-β signal transduction pathways. Furthermore, maintenance of fibrosis is likely mediated in part by dysregulation of CTGF expression.

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