IL-7 inhibits fibroblast TGF-β production and signaling in pulmonary fibrosis

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Based on studies by our group and others, we hypothesized that IL-7 may possess antifibrotic activities in an IFN-γ-dependent and independent manner. Here, we have evaluated the antifibrotic therapeutic potential of IL-7 in both in vitro and in vivo pulmonary fibrosis models. IL-7 inhibited both TGF-β production and signaling in fibroblasts and required an intact JAK1/STAT1 signal transduction pathway. IL-7–mediated inhibition of TGF-β signaling was found to be associated with an increase in Smad7, a major inhibitory regulator in the SMAD family. In the presence of IL-7, Smad7 dominant negative fibroblasts restored TGF-β–induced collagen synthesis, indicating that an IL-7–mediated increase in Smad7 suppressed TGF-β signaling. Consistent with these in vitro findings, recombinant IL-7 decreased bleomycin-induced pulmonary fibrosis in vivo, independent of IFN-γ. The antifibrotic activities of IL-7 merit further basic and clinical investigation for the treatment of pulmonary fibrosis.


Introduction

Idiopathic pulmonary fibrosis (IPF) is a devastating disease with less than a 50% five-year survival (1, 2). While steroids and other immunosuppressive agents serve as the standard treatment for IPF, these agents have proved inadequate (3). In most instances immunosuppressive agents do little to affect the course of the disease and have serious adverse side effects. Thus, novel therapeutic strategies are clearly needed. Recent published findings suggest novel treatment paradigms based on a more complete understanding of the pathogenesis of pulmonary fibrosis (3, 4).

IL-7 is a 25-kDa glycoprotein originally isolated from bone marrow stroma cells (5). IL-7 was originally defined as a pre-B lymphocyte growth factor and was subsequently found to augment the growth of T lymphocytes (6–8). We (9) and others (10–13) have documented that IL-7 can potently enhance T cell function and IFN-γ production. IL-7 synergizes with IL-12 in the induction of T cell proliferation, cytotoxicity, and IFN-γ release (11). In agreement with these findings are studies indicating that IL-7 plays a role in cell-mediated immune responses characteristic of type 1 cytokines (10). We have found that IL-7 downregulates macrophage (14), fibrosarcoma, and melanoma (15, 16) production of TGF-β. IL-7 has the capacity to downregulate the transcriptional rate of the TGF-β gene in murine macrophages in an IFN-γ-independent manner (14).

TGF-β is a critical fibrogenic factor in the development of pulmonary fibrosis. Based on the importance of TGF-β in the pathogenesis of pulmonary fibrosis, we speculated that the most effective therapies would be those that decrease both the production and the cellular effects of TGF-β. In the current study we investigate the role of IL-7 in TGF-β production and signaling and the potential for IL-7 as a new antifibrotic agent in the treatment of interstitial pulmonary fibrosis.

Methods

Cell culture. Pulmonary fibrosis fibroblasts (PFFs) were isolated from lung resection specimens obtained from patients with IPF. Normal fibroblasts (NFs) were isolated from patients with nonfibrotic diseases. U4A, U3A, and U4A/JAK1 cell lines were generously provided by George R. Stark (Cleveland Clinic Foundation, Cleveland, Ohio, USA). The cells were maintained in 5% CO2 in air as monolayers at 37°C in 75-cm2 tissue culture flasks containing 20 ml of DMEM supplemented with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine (JRH Biosciences, Lenexa, Kansas, USA).

Cytokines and antibodies. Human activated recombinant TGF-β1 (3.2 × 10^4 units/μg), recombinant human IL-7...
(10^5 units/µg), recombinant mouse IL-7 (5 × 10⁴ units/µg), recombinant human IFN-γ (2 × 10⁴ units/µg), and mouse anti-human IFN-γ monoclonal neutralizing antibody were obtained from R&D Systems Inc. (Minneapolis, Minnesota, USA). Anti-mouse IFN-γ monoclonal neutralizing antibody was purified by affinity chromatography from ascites of SCID mice, which were generated 3–4 weeks after intraperitoneal injection of 10⁶ R4-462 hybridoma cells per mouse (American Type Culture Collection, Rockville, Maryland, USA) (17).

Dominant negative Smad7. The retroviral vector containing a full-length Smad7 cDNA was generously provided by Rik Derynk (University of San Francisco, San Francisco, California, USA). The Smad7 dominant negative mutant was constructed by a 25–amino acid deletion at the COOH terminus of Smad7. After transfection to the PT-67 retroviral packaging cell line by a liposomal-mediated method (Effectene method; QIAGEN Inc., Valencia, California, USA), the supernatant containing high titer of retrovirus expressing dominant negative Smad7 was collected to transduce PFF cells.

Mice. Pathogen-free female C57BL/6 mice (6–8 weeks old) and CB17 SCID Beige mice (6–8 weeks old) were purchased from the Charles River Laboratories, Inc. (Wilmington, Massachusetts, USA) and maintained in the West Los Angeles Veterans Affairs Animal Research Facility.

Bleomycin-induced pulmonary fibrosis model. To induce pulmonary fibrosis, mice were treated with 0.15 U bleomycin in 25 µl 0.9% normal saline (NS) or 0.9% NS alone by intratracheal administration. One day before bleomycin instillation, mice were started on treatment with 50 µg of recombinant IL-7 or vehicle control followed by administration five times per week for 2 weeks by intraperitoneal injection. Fourteen days following bleomycin exposure, mice were euthanized and both lungs were removed for the determination of hydroxyproline, collagen, TGF-β, and IFN-γ content. Separate experiments assessed whether IL-7–mediated antifibrotic activities are IFN-γ–independent. Two days before bleomycin instillation, treatment was started with 100 µg of anti–IFN-γ neutralizing antibody or control antibody five times a week for 2 weeks by intraperitoneal injection to neutralize endogenous and IL-7–induced IFN-γ. In our previous studies we found that this dose of anti–IFN-γ neutralizing antibody had the capacity to neutralize >95% circulating IFN-γ in vivo (data not shown).

TGF-β and IFN-γELISA. Mouse anti-human TGF-β or IFN-γ monoclonal capture antibody and biotinylated detecting antibody pairs were obtained from R&D Systems Inc. Monoclonal anti–TGF-β antibody measures TGF-β1, 2, and 3. ELISA was performed based on a horseradish peroxidase method as described previously (14–16). OD of each sample after color development was determined with a microplate reader (Dynatech Laboratories, Chantilly, Virginia, USA) at 450 nm.

RT-PCR. The human Smad7 sense primer (5’-AGC ACA CCA GCT CGG GGT TGA T-3’) and antisense primer (5’-AAC GAT CTG CGC TCG TCC GCC G-3’) correspond to the coding regions 308–330 and 872–849 of the human Smad7 cDNA with amplified DNA size of 564 bp. The
housekeeping gene GAPDH was used as control for semiquantification. High-quality total RNA from 10^7 cells each of NF or PFF samples with or without IL-7 treatment (100 ng/ml) for 24 hours was prepared by the TRIzol method (GIBCO BRL, Life Technologies Inc., Grand Island, New York, USA). RT-PCR was performed at 42°C for 1 hour followed by 35 cycles of amplification using a PTC-100 programmable thermal cycler (MJ Research Inc., Watertown, Massachusetts, USA).

Collagen assay. Collagen assay was performed using the Sircol collagen assay method (Accurate Chemical & Scientific Corp., Westbury, New York, USA). Briefly, 1,000 µl of Sircol dye reagent was added to 100 µl of test samples or collagen standards and incubated at 25°C for 30 minutes. After centrifugation at 5,000 g for 5 minutes, the supernatants were drained off and discarded. One thousand µl of 0.5N NaOH was added to the collagen-bound dye pellet to release the bound dye into solution. The OD of each sample was determined with a microplate reader (Dynatech Laboratories) at 540 nm.

Hydroxyproline assay. Lungs were harvested on day 14 after bleomycin administration (18, 19). One-half milliliter of lung homogenate was digested in 1 ml of 6N HCl for 8 hours at 120°C. Five microliters of citrate/acetate buffer (5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid, pH 6.0) and 100 µl of chloramines-T solution (282 mg of chloramines-T, 2 ml of n-propanol, 2 ml of H2O, and 16 ml of citrate/acetate buffer, pH 6.0) were added to 5 µl of sample and incubated for 20 minutes. Next, 100 µl of Ehrlich’s solution (2.5 g of 4-(dimethyl-amino)benzaldehyde, 9.3 ml of n-propanol, 3.9 ml of 70% perchloric acid) was added to each sample and incubated for 15 minutes at 65°C. The OD was determined at 550 nm on a DU 640 spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA). Hydroxyproline (Sigma Chemical Co., St. Louis, Missouri, USA) concentrations from 0–10 µg/ml were used to construct a standard curve.

Statistical analysis. All in vitro results are representative of at least three independent experiments performed in triplicate. In vivo experiments were performed with 10 mice per group, which yields a power of 0.90 to detect any difference among the treatment groups (α = 0.05). Significance of differences between experimental and control values was calculated using the Student’s t test.

Results

IL-7 decreases TGF-β production by PFFs. Based on our previous studies in murine macrophages (14), murine fibrosarcomas (15), and human tumors (16), we hypothesized that IL-7 also would have the capacity to decrease fibroblast TGF-β production. In human primary cultures of pulmonary fibroblasts isolated from patients with IPF (20), we found that IL-7 (100 ng/ml) has the capacity to downregulate the production of TGF-β, which was quantified by ELISA using mAb’s recognizing TGF-β1, 2, and 3 (Figure 1a). IL-7, in contrast, does not alter TGF-β production in the primary cultures of normal fibroblasts derived from lung resection specimens from patients who do not have IPF (Figure 1b). Both PFFs and NFs express the IL-7 receptor (IL-7R). No difference was detected in the level of PFF and NF IL-7R expression when assessed by flow cytometry (data not shown). Because IFN-γ has previously been shown to decrease TGF-β production, we compared the capacities of IFN-γ and IL-7 to downregulate TGF-β and sought to determine whether their effects were additive. Both PFF and NF were tested and were devoid of IFN-γ in the culture supernatant (data not shown). We exposed PFF and NF to IL-7, IFN-γ (500 units/ml), IL-7 plus IFN-γ, or IL-7 plus anti–IFN-γ- monoclonal antibody (5 µg/ml) (Figure 1, c and d). Whereas NF responded only to IFN-γ by downregulating TGF-β production, PFF showed decreased TGF-β following exposure to either IL-7 or IFN-γ. As predicted, the addition of anti–IFN-γ antibody had no effect on IL-7–mediated downregulation of TGF-β because fibroblasts did not produce IFN-γ. The results suggest that IL-7 is signaling downregulation of TGF-β in an IFN-γ-independent manner and that PFF may have an accessory IL-7 signaling pathway or a different regulatory mechanism that is not operative in NF.

IL-7R signaling requires an intact JAK1/STAT1 signaling pathway. Activation of the JAK/STAT pathway has been implicated in IL-7R signaling (21, 22). Accordingly, we tested whether the capacity of IL-7 to decrease TGF-β production was mediated through JAK/STAT signaling. Using JAK1-deficient (U4A) and STAT1-deficient (U3A) mutant fibroblast cell lines, we observed that IL-7 had no effect on TGF-β production in the U4A and U3A cells, whereas U4A/JAK1 fibroblasts responded to IL-7 by downregulating TGF-β production in a dose-dependent manner in U4A/JAK1 cells (P < 0.01 at 50–400 ng/ml IL-7).
dose-dependent manner (Figure 2). These findings indicate that the downregulation of TGF-β production in fibroblasts by IL-7 requires an intact JAK1/STAT1 signal transduction pathway. In contrast to fibroblasts from nonfibrotic lungs, the U4A/JAK1 fibroblast cell line shows decreased TGF-β in response to IL-7 and, in this regard, is similar to PFFs.

IL-7 decreases TGF-β–induced collagen synthesis. In addition to decreasing TGF-β production, another important contribution for a cytokine used therapeutically to modulate the fibrotic response would be the capacity to inhibit the effects of TGF-β signaling (21, 22). TGF-β is known to stimulate both fibroblast proliferation (23, 24) and collagen deposition (25). To test whether IL-7 could modulate TGF-β–induced collagen synthesis, we exposed PFF and NF to activated recombinant TGF-β1 alone (10 ng/ml) or TGF-β plus IL-7 (100 ng/ml). When used at this concentration, TGF-β did not significantly impact fibroblast proliferation at 24 hours in either NF or PFF (data not shown). When stimulated with TGF-β, PFF but not NF showed enhanced collagen production (Figure 3). Following preincubation in IL-7 (100 ng/ml) for 2 hours before the addition of TGF-β1, there was a marked reduction in the capacity for TGF-β to elicit fibroblast collagen synthesis in PFF (Figure 3). Interestingly, IL-7 had no effect on the constitutive level of collagen synthesis. This suggests that IL-7 inhibits collagen synthesis by blocking TGF-β signaling in PFF.

IL-7 inhibits TGF-β signaling through the induction of Smad7. TGF-β signaling and regulation are mediated by a family of SMAD proteins (21, 22). Among the SMAD family, Smad7 is the major inhibitor of TGF-β signaling. Previous studies indicate that IFN-γ has the capacity to regulate Smad7 through the JAK/STAT pathway (21). Based on our observation that IL-7 also mediates its effects in PFF through the JAK/STAT pathway, we hypothesized that IL-7 interfered with TGF-β signaling and, therefore, decreased TGF-β–induced collagen synthesis by enhancing Smad7 expression in PFF. As shown in Figure 4a, IL-7 increased Smad7 in pulmonary fibroblasts. In order to determine whether the IL-7–induced increase in Smad7 expression was the cause of IL-7–mediated inhibition of collagen synthesis, PFF were transduced with a Smad7 dominant negative construct. The levels of collagen synthesis in response to IL-7 were evaluated in both untransduced fibroblasts and the Smad7 dominant negative–transduced (Smad7DN-transduced) cells. In the presence of IL-7, Smad7DN-transduced fibroblasts maintained high-level TGF-β–induced collagen synthesis, indicating that an IL-7–mediated increase in Smad7 led to inhibition of TGF-β signaling (Figure 4b).

IL-7 inhibits bleomycin-induced pulmonary fibrosis in vivo. Based on the capacity of IL-7 to downregulate TGF-β production and block TGF-β signaling in human PFF, we tested whether recombinant IL-7 would limit the...
development of bleomycin-induced pulmonary fibrosis in vivo. Consistent with our in vitro findings, recombinant IL-7 administration (5 µg/mouse five times per week for 2 weeks) by intraperitoneal injection decreased bleomycin-induced pulmonary fibrosis, as indicated by a decrease in hydroxyproline and collagen content, decreases TGF-β, and increases IFN-γ in bleomycin-treated lung homogenates. *P < 0.01 IL-7 + bleomycin compared with bleomycin alone.

**Discussion**

The TGF-β superfamily is a group of multifunctional regulatory peptides that have a broad array of activities in cell growth and differentiation (23–34). TGF-β plays a pivotal role in tissue fibrosis (23–34), including pulmonary fibrosis (23–26, 31–34). Within the normal lung, TGF-β1 is the most abundant isoform and is mostly expressed in interstitial fibroblasts and bronchial epithelial cells (24, 25, 31–33). Within lungs developing pulmonary fibrotic responses, TGF-β1 is found to be overexpressed in a broad range of cells including fibroblasts, macrophages, and epithelial and endothelial cells (23–34). The pathogenesis of pulmonary fibrosis includes the deterioration of the normal homeostatic mechanisms regulating the equilibrium between the synthesis and breakdown of the ECM (24). Overexpression of TGF-β contributes to the dysregulation of the normal homeostasis at multiple levels: it enhances synthesis and deposition of ECM components including collagen and can also alter the balance of matrix metalloproteinases and their inhibitors (24, 25, 30–34). Based on the importance of TGF-β in the pathogenesis of pulmonary fibrosis, in the current study IL-7 was chosen for evaluation, because we have previously found that IL-7 downregulates macrophage (14), fibrosarcoma, and melanoma (15, 16) production of TGF-β.

The current findings indicate that recombinant IL-7 significantly decreases TGF-β production in PFF but using anti-IFN-γ neutralizing antibody (100 µg/mouse five times per week for 2 weeks) administered by intraperitoneal injection and assessed hydroxyproline and collagen levels. The results indicated that IL-7 decreased bleomycin-induced pulmonary fibrosis in an IFN-γ-independent manner (Figure 6).
not in NF. Because IL-7R expression did not differ between PFF and NF cells, we speculate that downstream regulatory signaling events account for this difference in IL-7 responsiveness. Further studies will be necessary to define these differences. As is documented for several other cytokines, IL-7 has previously been found to signal by the JAK1/STAT1–dependent pathway in lymphocytes (35). Based on these previous studies, we tested whether IL-7 could mediate a decrease in fibroblast TGF-β in the absence of JAK1/STAT1. Our current findings document, for the first time to our knowledge, that JAK1/STAT1– dependent signaling is operative for IL-7 in human fibroblasts.

JAK/STAT–dependent signaling in fibroblasts has been previously found to be critical for the capacity of IFN-γ to upregulate Smad7 and inhibit TGF-β signaling (21). IFN-γ signaling rapidly increases the expression of Smad7, causing the inhibition of Smad3 phosphorylation and subsequently loss of TGF-β signaling to the nucleus (21, 22). These findings indicate a mechanism of transmodulation between the STAT and SMAD signal transduction pathways. In our current studies, in addition to the capacity to downregulate TGF-β production, IL-7 was found to potently induce Smad7 and subsequently block TGF-β signaling. The functional manifestation of this blockade in TGF-β signaling is demonstrated by a profound decrease in the TGF-β–induced collagen synthesis in PFF. IFN-γ is known to decrease both TGF-β production and signaling and has shown promise in preliminary clinical trials (4). Although IL-7 is known to induce IFN-γ, our current study indicates that IL-7 mediates potent antifibrotic responses both in vitro and in vivo in an IFN-γ–independent manner.

A variety of models have been used to assess potential therapeutic interventions for pulmonary fibrosis (18–20, 32, 33, 36–46). In this study we used the bleomycin model because of its widespread use for this purpose (18–20, 32, 33, 36–39, 41–46). However, bleomycin has been known to induce acute lung injury, thus initiating the process of bleomycin-induced fibrosis (39). Although the bleomycin model does not fully replicate the human disease that is clinically recognized as IPF, we chose to use this model because bleomycin induces a TGF-β–dependent induction of pulmonary fibrosis that is accompanied by increased collagen and hydroxyproline synthesis (18–20, 32, 33, 37, 41, 42, 44–46). For example, studies indicate that administration of anti–TGF-β antibodies (32), soluble TGF-β receptors (41), the TGF-β inhibitor decorin (43, 44), or Smad7 gene transfer (37) limits bleomycin-induced pulmonary fibrosis, thus strongly implicating a pathogenetic role for TGF-β in this model. Consistent with our in vitro findings in human fibroblasts derived from pulmonary fibrosis biopsies, IL-7 inhibited pulmonary fibrosis in vivo in the bleomycin model.

Although investigators have shown that TGF-β can impact the phenotype and function of normal fibroblasts, studies also demonstrate marked differences between normal and fibrosis fibroblasts (26–30). The differences in TGF-β–induced collagen synthesis by NF and PFF may be due to a differential response of these cell populations to this cytokine and are consistent with previously reported observations (26–30). In our current study, recombinant TGF-β did not affect collagen synthesis in normal fibroblasts under the conditions and concentrations evaluated. Because activated recombinant TGF-β1 was used in these studies, potential differences in TGF-β activation state could not account for these observations. Our findings are consistent with previous investigations that revealed marked differences in TGF-β–dependent responses in fibroblasts from different developmental or pathogenic states (27–30). The precise mechanism of the differential response to TGF-β by PFF and NF remains unclear and requires further investigation.

The current study was designed to assess the effectiveness of IL-7 as a potential antifibrotic agent and to begin to understand the capacity of this cytokine to regulate TGF-β production and signaling. The full clinical relevance of these findings will require further investigation, including an assessment of the time course of IL-7 efficacy following bleomycin exposure. Novel treatment paradigms are being developed based on a more complete understanding of the pathogenesis of IPF (1–4). In marked contrast to the current forms of therapy for IPF that use steroids and immunosuppressive agents, IL-7 potently enhances cell-mediated immunity. In addition to its previously documented capacity to induce IFN-γ, we report here that IL-7 downregulates both TGF-β production and signaling in pulmonary fibroblasts. Our results suggest that IL-7 is a potentially therapeutic cytokine for IPF.

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