Interleukin-13 Modulates Collagen Homeostasis in Human Skin and Keloid Fibroblasts

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Accepted for publication November 8, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

Interleukin (IL)-13 has been implicated in the pathogenesis of various diseases characterized by fibrosis. We describe the effects of IL-13 on collagen homeostasis from normal (NF) and keloid (KF) fibroblasts and compare these effects with those of IL-4 and transforming growth factor (TGF)-β. Total collagen generation was up-regulated in NF after 48 h of stimulation by IL-13; in KF, IL-13 stimulated a more rapid collagen response. The kinetics and magnitude of collagen generation induced by IL-13 were equivalent to those induced by similar concentrations of IL-4 and TGF-β. Collagen type I production paralleled total collagen generation from both NF and KF; however, IL-4-induced collagen type I and total collagen production from KF was more transient than that induced by either IL-13 or TGF-β. Procollagen 1α1 gene expression was induced in KF by stimulation with IL-13 for 24 h. Moreover, IL-13 was unique among these three cytokines in its ability to induce gene expression for procollagen 3α1. Finally, IL-13 inhibited IL-1β-induced matrix metalloproteinase (MMP)-1 and MMP-3 production and enhanced tissue inhibitor of metalloproteinase (TIMP)-1 generation from NF; although similar effects were observed with IL-4, TGF-β, transiently enhanced MMP-1 and MMP-3 generation without affecting TIMP-1. In KF, IL-13 and IL-4 inhibited MMP-3, whereas TGF-β enhanced MMP-3; TIMP-1 was unaffected by any of the three cytokines. These data demonstrate both the profibrotic effects of IL-13 on collagen homeostasis and the potential differential regulation of collagen homeostasis in fibroblast subtypes by IL-13.

Tissue remodeling of the extracellular matrix (ECM) is an essential and dynamic process associated with physiological and pathological responses. Remodeling involves both the degradation and clearance of ECM components, as well as the production and deposition of newly synthesized components; the balance of these processes results in either preservation or alteration of the structure and functions of the supported tissue (Liu and Connolly, 1998). Resorption of the ECM is mediated predominantly by the matrix metalloproteinases (MMPs), whereas generation of ECM is predominantly achieved through the production of collagen (Mauch, 1998). Because excessive degradation of ECM may characterize pathological states such as arthritis (Cawston, 1998) and tumor invasion (Airola et al., 1997) and increased generation of ECM underlies fibrotic diseases (Jimenez et al., 1996), both processes are strictly regulated by complex networks of cellular and molecular interaction. Mediators such as cytokines released by resident cells or infiltrating leukocytes may play a major role in ECM homeostasis (Hatamochi et al., 1994). Interestingly, although T helper cells may be subclassified as type 1 (Th1) or type 2 (Th2) based largely on mutually exclusive cytokine repertoires and individual T cell subclasses may underlie specific disease processes, both Th1- and Th2-mediated diseases are associated with fibrosis (Abbás et al.; 1996). Although various cytokines have been described as “profibrotic” (interleukin (IL)-4, IL-6, IL-11, and transforming growth factor (TGF)-β) (Postlethwaite, 1995; Tang et al., 1996; Minshall et al., 1997; Coker and Laurent, 1998), few have been described with the potential to be major constituents of both Th1 and Th2 responses in humans.

IL-13 is a four-helix bundle cytokine located adjacent to IL-4 on chromosome 5q31. IL-13 shares several structural and functional characteristics with IL-4; however, unlike IL-4, IL-13 is produced by both human Th1 and Th2 cells, its generation is prolonged after cellular activation, and the amount of IL-13 produced is >10-fold that of IL-4 (Essayan et al., 1996; de Vries, 1998). Increased levels of IL-13 (~1000 times higher than levels of IL-4) have been detected in the serum of patients affected by systemic sclerosis (a Th1-mediated disease) (Hasegawa et al., 1997) and in the bronchoal-

ABBREVIATIONS: ECM, extracellular matrix; KF, keloid fibroblast; MMP, matrix metalloproteinase; NF, normal fibroblast; Col1α1, procollagen 1α1; Col3α1, procollagen 3α1; OH-Pro, hydroxyproline; IL, interleukin; TGF, transforming growth factor; PCR, polymerase chain reaction; RT, reverse transcription; TIMP, tissue inhibitor of metalloproteinase.
veolar lavage of patients affected by asthma (a Th2-mediated disease) and different forms of pulmonary fibrosis (Huang et al., 1995; Hancock et al., 1998). However, the role of IL-13 in tissue remodeling in humans has not been investigated. To better understand the potential contributions of IL-13 to ECM remodeling, we studied the effects of IL-13 on 1) total and subtype-specific collagen generation, 2) production of collagenase (MMP-1) and stromelysin-1 (MMP-3), and 3) production of the endogenous MMP inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1), each in one normal fibroblast (NF) and one abnormal (keloid) fibroblast (KF) cell line. Throughout this study, we compared the effects of IL-13 with those of the “profibrotic” Th2/Th3 cytokines, IL-4 and TGF-β1.

Materials and Methods

Cell Culture and Cytokine Stimulation. Human KP (CRL 1762) and human skin fibroblasts (CRL 7315) were purchased from American Type Culture Collection (Rockville, MD). Fibroblasts were grown in 175-cm² tissue culture flasks with Dulbecco’s modified Eagle’s medium containing 15% FBS supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Gaithersburg, MD) and incubated at 37°C in an atmosphere of air and 100% relative humidity. After reaching confluence, cells were harvested using Dulbecco’s PBS containing 0.25% trypsin/0.5% EDTA and subcultured under the same conditions (Sigma Chemical Co., St. Louis, MO). Only early passage cells (third through seventh) were used in these experiments. At 48 h preceding experiments, fibroblasts were made quiescent by maintenance in low serum (0.1% FBS). Cells were then incubated in fresh Dulbecco’s modified Eagle’s medium containing 0.1% FBS and 50 µg/ml acriflavine (Sigma Chemical Co.) in the presence and absence of specified cytokine (Peprotech, Cherry Hill, NJ, and Biosource International, Camarillo, CA) for specified periods of time (24, 48, or 72 h). Experiments to study the resolution of collagen generation induced by cytokine were initiated as earlier and incubated for 48 h in the presence and absence of cytokine; cells were then washed, and supernatant and cell wash were combined for analysis. These samples represent 0 h. Cells were then incubated with fresh media, and these supernatant-plus-cell wash samples were analyzed at 24 and 48 h. Each individual experiment was performed in duplicate.

Hydroxyproline (OH-Pro) Release Assay. The evaluation of OH-Pro content allows estimation of total collagen content, because OH-Pro produced by fibroblasts is nearly all incorporated into collagen (Kreis and Vale, 1993). OH-Pro content was assessed by a modification of the method of Woessner (1961). Briefly, samples were collected and hydrolyzed for 18 h in the presence of an equivalent amount of hydrochloric acid (12 N) at 100°C. After the evaporation of the acid, the pellet was resuspended in Tris buffer, the OH-Pro was oxidized in Chloramine-T, and the resultant pyrrole reacted with p-dimethylaminobenzaldehyde in the presence of perchloric acid (Sigma Chemical Co.). The absorbance of each sample was analyzed by spectrophotometry at 557 nm (Beckman Scientific, Fullerton, CA), and the OH-Pro content was quantified relative to a standard curve generated using synthetic OH-Pro (Sigma Chemical Co.).

RNA Isolation and Reverse Transcription (RT)-Polymerase Chain Reaction (PCR). RNA isolation and RT-PCR were performed using a modification of our previously published method (Essayan et al., 1997). After culture, NFs or KFs were washed free of medium with PBS and subjected to RNA isolation by the RNazolB technique (Tel-Test, Inc., Friendswood, TX) according to the manufacturer’s instructions. Diethylpyrocarbonate-treated water without SDS was used for the final resuspension step; RNA was stored at −80°C. RT was performed in the presence of 5 mM magnesium chloride, oligo d(T)₁₆ primer, and murine leukemia virus reverse transcriptase according to the manufacturer’s instructions (Perkin-Elmer Cetus, Foster City, CA) on a thermocycler (Technie, Cambridge, UK). PCR was performed at sub saturating cycle numbers using Taq polymerase (1–2.5 U/reaction) and target-specific primers (0.2–1 µM/primer) designed in our laboratory and produced and purified at the Johns Hopkins DNA Core Facility. Normalization of RNA was achieved by RT-PCR for the constitutive marker gene β-actin at sub saturating cycle numbers. Strict RNase-free conditions were maintained throughout. The specificity of amplification was confirmed by direct sequencing of the RT-PCR amplification products after cloning into TA vector (Promega Corp., Madison, WI) according to the manufacturer’s instructions. All sequencing was performed at the Johns Hopkins DNA Core Facility. All PCR products were visualized by ethidium bromide-stained gel electrophoresis and photographed.

Western Blot Analysis of Type-Specific Collagen Generation. Duplicate samples were subjected to 4 to 20% acrylamide gradient SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to nitrocellulose (200 mA for 20 h in a Western blotting apparatus water cooled to 4°C; Bio-Rad Laboratories, Hercules, CA), blocked with 10% powdered milk (Carnation) in PBS with 0.1% Tween 20, and reacted with first antibody (LF67, rabbit anti-human collagen 1, generously provided by Dr. L. W. Fisher, National Institutes of Health) at a 1:20,000 dilution. The blots were reacted with second antibody (goat anti-rabbit horseradish peroxidase; Pierce Chemical Co., Rockford, IL), developed for chemiluminescence using enhanced chemiluminescence reagents (Pierce Chemical Co.) and analyzed with a digital scanner 2400 (Relays, Milpitas, CA) using National Institutes of Health Image 1.59 software to determine band intensity and migration position. Band intensities were normalized to total protein content and expressed as stimulation index.

MMP and TIMP Protein Secretion Assays. MMP and TIMP protein secretion were assessed by enzyme-linked immunosorbent assay, using Biotrak cellular communication assays (Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to the manufacturer’s instructions, with World Health Organization standards provided by the company. Fibroblasts were incubated in DMEM containing 5% FBS at a density of 1 × 10⁵/ml in multiwell tissue culture plates. These cultures were treated with specified cytokine for specified periods of time. TIMP production was assessed in culture supernatants collected after incubation with the designated cytokine. MMP production was assessed in culture supernatants after 24- or 48-h preincubation with the designated cytokine and a subsequent 48-h incubation with IL-1α (5 ng/ml) (Peprotech); the duration of preincubation had no effect on the results. Supernatants were stored at −20°C until assayed. Dilutions of samples, when necessary, were performed in culture medium. All standards and samples were tested in duplicate; most samples were analyzed at two different dilutions and compared for internal consistency.

Statistical Analysis. Mean and S.E. values, as well as t test comparisons, were derived using StatView (BrainPower, Inc., Calabassas, CA) on a Macintosh computer. P values are paired, two-tailed; P ≤ .05 is considered statistically significant.

Results

Stimulation of Collagen Generation from NFs and KFs by IL-4, IL-13, and TGF-β₁. Figure 1 depicts total collagen secretion from NF (expressed as ng OH-Pro×10⁵ cells) induced by IL-4 (10 and 100 ng/ml), IL-13 (10 and 100 ng/ml), and TGF-β₁ (5 ng/ml), each at 24, 48, and 72 h of stimulation. Although none of the three cytokines induced collagen generation at 24 h of stimulation, all three induced significant increases in collagen secretion after 48 h of stimulation. Interestingly, the amount of collagen secretion reached a plateau after 48 h of stimulation for all three.
cytokines. There were no significant differences between the 10 and 100 ng/ml concentrations of IL-4 and IL-13. Although 1 ng/ml IL-4 or IL-13 induced minor increases in collagen generation from NF at 48 h, 0.1 ng/ml IL-4 or IL-13 was ineffective at inducing collagen generation from NF (data not shown).

Figure 2 depicts total collagen secretion from KF (also expressed as ng OH-Pro/2 × 10^5 cells/ml) induced by the same three cytokines at the same three time points. KF produce approximately three to five times the amount of collagen compared with NF. Again, all three cytokines induced significant increases in collagen secretion. This increase was apparent after only 24 h of stimulation in the KF stimulated with IL-4 or IL-13; no stimulation was evident at 6- and 12-h time points (data not shown). Collagen generation continued to increase as late as 72 h after stimulation. Again, there were no significant differences between the 10 and 100 ng/ml concentrations of IL-4 and IL-13; although 1 ng/ml IL-4 or IL-13 induced minor increases in collagen generation from KF at 24 and 48 h, 0.1 ng/ml IL-4 or IL-13 was ineffective at inducing collagen generation from KF (data not shown). Thus, IL-4- and IL-13-induced collagen generation from KF is more rapid and of greater magnitude than that from NF; however, there are no apparent differences in the dose-response relationships of IL-4- and IL-13-induced collagen generation between these cell lines.

Resolution of Collagen Generation from NF and KF after Exposure to IL-4, IL-13, and TGF-β1. Figure 3 depicts total collagen generation after 48 h of stimulation with cytokine and again 24 h after removal of cytokine (24 h chase) in NF (Fig. 3A) and KF (Fig. 3B), respectively. NF continued to display enhanced collagen production 24 h after removal of IL-4, IL-13, or TGF-β1; the level of collagen production was not significantly changed. However, collagen production 48 h after cytokine removal returned to control values (data not shown); intermediate time points were not evaluated. KF continued to display enhanced collagen production 24 h after removal of IL-13 or TGF-β1; however, collagen production was back to control values 24 h after removal of IL-4. Again, the level of collagen production 48 h after cytokine removal returned to control values for all three cytokines (data not shown). Thus, collagen production induced by IL-4 may display more transient kinetics compared with IL-13 or TGF-β1.

Stimulation of Type-Specific Collagen Generation from NF and KF by IL-4, IL-13, and TGF-β1. Figure 4 depicts the β-actin-, procollagen 1α1 (Col1α1)-, and procollagen 3α1 (Col3α1)-specific RT-PCR amplification products from experiments in which KF were cultured for 24 h with media alone or with various cytokines as indicated. Adequate normalization of RNA for each sample was confirmed by the equality of RT-PCR amplification products for β-actin gene expression at subsaturating cycle number (30 cycles); the specificity of amplification was confirmed by direct sequenc-
ing of RT-PCR amplification products. Exposure to 10 or 100 ng/ml IL-13 increased the expression of Col1α at 24 h of stimulation (Fig. 4, top); this enhancement was maintained at both 48 and 72 h of stimulation. IL-4 and TGF-β, induced similar early increases in Col1α gene expression (data not shown). Figure 4, bottom, demonstrates the induction of Col3α gene expression exclusively by IL-13 at 24 h after stimulation; the induction of Col3α gene expression by IL-13 was significantly decreased at 48 h and was not evident at 72 h (data not shown). These findings suggest that IL-13
induces a unique, rapid, but transient increase in Coll3α and a more sustained increase in Coll1α gene expression in KF. Similar data were obtained from NF.

Figure 5 depicts collagen type I protein secretion (measured by Western blot densitometry as stimulation index relative to media controls) induced by IL-4, IL-13, or TGF-β1 at 24 and 48 h from NF (Fig. 5A) and KF (Fig. 5B), respectively. All three cytokines induced a significant rise in collagen type I generation from NF at 48 h of stimulation; however, only IL-13 and TGF-β1 showed significant enhancement at 24 h. By 72 h, collagen type I stimulation from NF had returned to control levels (data not shown). These data correspond well with the plateau of effect seen with total collagen generation (Fig. 1). In KF, all three cytokines induced a significant rise in collagen type I generation at 24 h; in the cases of IL-13 and TGF-β1, this induction was maintained at 48 (and 72 h). However, the early rise seen with IL-4 showed a small but significant decline by 48 h; this decline continued at 72 h (data not shown).

Modulation of MMP and TIMP Generation from NF and KF by IL-4, IL-13, and TGF-β1. Table 1 shows the percent change (±S.E.) in MMP-1, MMP-3 (each relative to IL-1β-stimulated cultures, corrected for background with media alone), and TIMP-1 (relative to cultures with media alone) induced by preincubation of NF and KF with IL-4, IL-13, or TGF-β1, for 24 or 48 h. MMP production in the absence of IL-1β stimulation was minimal; IL-1β had no effect on TIMP-1 generation. In NF, IL-4 and IL-13 induced significant inhibition of MMP-1 and MMP-3; TGF-β1 induced a transient up-regulation of MMP-1 and MMP-3 under the same conditions. TIMP-1 was up-regulated by all three cytokines after 48 h. In KFs, TGF-β1 induced marked and sustained up-regulation of MMP-3, whereas IL-4 and IL-13 both induced significant down-regulation of MMP-3. TIMP-1 remained unchanged by preincubation with these three cytokines. Finally, the induction of MMP-1 production by IL-1β was minimal from KF, obviating our ability to measure cytokine effects on its regulation (data not shown). These findings suggest both differential regulation of MMP generation by cytokines and differential regulation of cytokine-induced MMP generation between NF and KF. Finally, these data suggest a primary role for TGF-β1 in tissue repair and for IL-4 and IL-13 in tissue fibrosis, as summarized in Table 2.

Discussion

Tissue remodeling involves responses to exogenous and endogenous signals that promote either restoration of normal tissue architecture or generation of abnormal architecture. A central component in the generation of abnormal architecture is expansion of the ECM by collagen deposition, resulting in fibrosis. A number of inflammatory mediators have been implicated in the generation of fibrosis, including epidermal growth factors, fibroblast growth factors, insulin-like growth factors, platelet-derived growth factors, and cytokines such as IL-4, IL-6, IL-11, and TGF-β (Duncan and Berman, 1991; Kovacs and DiPietro, 1994; Tang et al., 1996; Minshall et al., 1997; Liu and Connolly, 1998). However, we questioned whether any of these mediators were elaborated in sufficient quantity during both Th1 and Th2 responses to account for fibrosis in both situations. IL-13 is a product of both human Th1 and Th2 cells that displays both prolonged kinetics of expression and 10- to 1000-fold molar excess of secretion (Th2 production of IL-13 >10 ng/ml after antigen stimulation) compared with IL-4 (Essayan et al., 1996; Akdis et al., 1997; de Vries, 1998). IL-13 is also a product of other resident tissue and inflammatory cells, including basophils (Redrup et al., 1998). IL-13 induces the expression of adhesion molecules, MCP-1, and IL-6 from pulmonary fibroblasts (Doucet et al., 1998). Interestingly, fibroblast cell lines express IL-13Rα1, IL-13Rα2, and IL-4Rα but not the IL-2R common γ-chain necessary for IL-4-specific receptor signaling (Feng et al., 1998; Murata et al., 1998). IL-13 has been implicated in the pathogenesis of a number of diseases associated with fibrosis, including atopic dermatitis, asthma, systemic sclerosis, and idiopathic pulmonary fibrosis (Huang et al., 1995; Hamid et al., 1996; Hasegawa et al., 1997; Hancock et al., 1998; Van der Pouw Kraan et al., 1998); recent data have demonstrated the generation of pulmonary fibrosis in IL-13 transgenic mice (Zhu et al., 1999). In this study, we sought to define the pharmacological effects of IL-13 on collagen homeostasis by using two different human fibroblast lines and potentially physiologically relevant quantities of cytokine; we performed parallel studies with IL-4 and TGF-β1 for comparison.

Our data demonstrate at least three potential roles for IL-13 in the generation and progression of fibrosis. First, IL-13 induces marked up-regulation of total collagen and type I collagen generation from both NFs and KFs; IL-13 is equipotent with IL-4 and TGF-β1 in this capacity. This observation assumes potentially greater importance in light of
the kinetics and quantity of IL-13 generation and the number of potential sources of this cytokine in sites of fibrosis. The local in vivo levels of IL-13 at sites of inflammation, equivalent to those used in these studies and readily produced by antigen-stimulated Th2 clones, may greatly exceed those used in these studies and readily produced by potential sources of this cytokine in sites of fibrosis. The potential role of IL-13 should be carefully considered in the pathogenesis and treatment of inflammatory and fibrosing diseases.

In conclusion, we demonstrated a role for IL-13 in the dysregulation of collagen homeostasis in sites of inflammation.

A few additional points deserve note. First, minimal (<1%) cellular proliferation was seen under the conditions of culture for collagen generation, regardless of the absence or presence of exogenous cytokine. Second, separate assessment of the cell layer pool collagen content for each culture condition at each time point revealed a low and constant level without significant differences for any of the samples (the cell layer pool represents intracellular collagen as well as collagen bound in the ECM). Third, we were unable to detect either precipitated collagen or collagen adherent to the plastic culture plates; thus, secreted collagen is representative of total collagen production. Finally, although the incremental changes in collagen homeostasis depicted in this study are small, the pathophysiological process of fibrosis is slow and cumulative, so the changes induced by IL-13 are likely to be biologically relevant.

Animal models of IL-4 and TGF-β overexpression have been reported; although pulmonary expression of IL-4 in transgenic mice resulted in little or no pulmonary fibrosis, an adenoviral transfection model for the pulmonary expression of active TGF-β resulted in the marked deposition of matrix and scar formation in rat lung (Rankin et al., 1996; Sime et al., 1997, 1998). These findings would not have been predicted from our data; clearly, species differences and additional pathways active in vivo may modulate the expected pharmacological effects of specific cytokines on collagen homeostasis. For example, TGF-β may act in part through the induction of connective tissue growth factor, causing a secondary enhancement of collagen synthesis and deposition (Mutsaers et al., 1997). However, histological findings from a transgenic mouse model of pulmonary IL-13 overexpression showed subepithelial collagen deposition, corresponding with our data (Zhu et al., 1999); additional reports further implicate IL-13 as a key regulator in the pathogenesis of allergic inflammation (Grunig et al., 1998; Wills-Karp et al., 1998). Finally, a selective inhibitor of IL-13 recently demonstrated efficacy in blocking Th2-driven hepatic fibrosis in mice infected with Schistosoma mansoni (Chiarabone et al., 1999); a similar level of blockade was evident in wild-type and IL-4-deficient mice. Thus, although the actual profibrotic signal intensity of IL-13 relative to other cytokines (e.g., IL-4) would be a function of the relative levels of cytokine, receptor expression, and receptor occupancy, these data suggest a predominant role for IL-13 in the clinical expression of fibrosis. The potential role of IL-13 should be carefully considered in the pathogenesis and treatment of inflammatory and fibrosing diseases.

Table 1

<table>
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<td>↑</td>
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<tr>
<td>IL-13</td>
<td>↑</td>
<td>↑</td>
<td>Remodeling/Repair</td>
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<tr>
<td>TGFβ1</td>
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<td>↑</td>
<td>Fibrosis</td>
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Table 2

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<td>−13 + 10</td>
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