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Human Pulmonary Fibroblasts Exhibit Altered Interleukin-4 and Interleukin-13 Receptor Subunit Expression in Idiopathic Interstitial Pneumonia

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Abnormal proliferation of pulmonary fibroblasts is a prominent feature of chronic pulmonary fibrotic diseases such as idiopathic interstitial pneumonia (IIP), but it is not presently clear how this proliferative response by lung fibroblasts can be therapeutically modulated. In the present study, we examined whether it was possible to selectively target primary human pulmonary fibroblasts grown out of surgical lung biopsies (SLBs) from IIP patients based on their expression of interleukin-4 receptor (IL-4R) and IL-13R subunits. Pulmonary fibroblast lines cultured from patients with the severest form of IIP, namely usual interstitial pneumonia, exhibited the greatest gene and protein expression of IL-4Rα, IL-13Rα1, and IL-13Rα2 compared with primary pulmonary fibroblast lines grown from other IIP SLBs and normal SLBs. When exposed to increasing concentrations of a chimeric protein comprised of human IL-13 and a truncated version of *Pseudomonas* exotoxin (IL13-PE), the proliferation of primary usual interstitial pneumonia fibroblasts was inhibited to a much greater extent compared with fibroblast lines from nonspecific interstitial pneumonia and respiratory bronchiolitis/interstitial lung disease patient groups. Fibroblasts from normal patients exhibited minimal susceptibility to the cytotoxic effect of IL13-PE. IL13-PE-mediated targeting of IIP fibroblasts was dependent on their expression of IL-4Rα and IL-13Rα2. Thus, these data suggest that the abnormal proliferative properties of human lung fibroblasts from certain IIP patient groups can be modulated in a manner that is dependent on the IL-4 and IL-13 receptor subunit expression by these cells. *(Am J Pathol 2004, 164:1989–2001)*

Considerable research effort has been directed toward elucidating the cellular and molecular mechanisms through which fibroblasts are triggered and remain activated during progressive, fibrosing disease of the lung parenchyma.[1–3] Usual interstitial pneumonia (UIP) is a severe form of pulmonary fibrosis, which is fatal in most patients because of slowly increasing dyspnea, restrictive lung dysfunction, and impaired gas exchange.[4] Because treatment options remain unsatisfactory for this and other forms of idiopathic interstitial pneumonia (IIP), the cytokine milieu that leads to sustained activation of the pulmonary fibroblast has been a focus of ongoing investigation. In this schema, it is proposed that an immune response dominated by interferon (IFN)-γ and other Th1-type cytokines such as interleukin (IL)-12 and IL-18 prevents overt fibroblast activation and pulmonary fibrosis.[5] Conversely, an immune response dominated by Th2-type cytokines such as IL-4 and IL-13 influence fibroblast activity to such an extent that the phenotype of this cell is markedly altered and it becomes a motile cell[6] with unregulated proliferative and synthetic cell in the lung.[5,7,8] Consistent with this schema, increased expression of Th2-type cytokines has been reported in lung biopsies[9] and isolated alveolar macrophages[10,11] from patients with IIP. More recent data demonstrating a beneficial effect of IFN-γ1b treatment in IIP patients who were not responsive to corticosteroids[12] further suggests that alteration of the cytokine balance within the fibrotic lung may prove therapeutically important.

IL-13 has been shown to have comparable effects with IL-4 and transforming growth factor-β in augmenting col-

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lagen generation and homeostasis in normal and keloid fibroblasts.[13] In addition, both IL-4 and IL-13 have been shown to increase α-smooth muscle actin expression thereby activating fibroblasts to become myofibroblasts.[14] IL-4 and IL-13 increased production of collagen and modified the equilibrium between MMP-1 and its inhibitor, TIMP-1.[15] Although it has been shown that cultured human fibroblast lines express IL-4 and IL-13 receptor subunits,[16–24] to date a detailed characterization of the expression of these subunits in IIP primary pulmonary fibroblasts has not been reported. The IL-4 and IL-13 receptors are multimeric and share the IL-4Rα subunit. Two types of IL-4 receptors have been described and include the IL-4-specific type 1 IL-4R comprised of the IL-4Rα and the γ-chain, and the IL-4/IL-13 binding type 2 IL-4R comprised of the IL-4Rα and IL-13Rα1 subunits. Computer modeling has shown that at least two binding sites for IL-4 and IL-13 are present on synovial fibroblasts suggesting that these cells express at least two different IL-4/IL-13 receptors.[22] This model is consistent with previous data showing that both IL-4 and IL-13 share common[21,24] and divergent[19] signal transduction pathways leading to the up-regulation in the expression of adhesion molecules and proinflammatory cytokines and chemokines.[19,20] However the presence of the high-affinity IL-13Rα2 subunit appears to act as a nonsignaling or decoy receptor for IL-13, but does not bind IL-4.[20]

In the present study, we examined primary fibroblasts lines grown from surgical lung biopsies (SLBs) from IIP, including UIP, nonspecific interstitial pneumonia (NSIP), respiratory bronchiolitis/interstitial lung disease (RBILD), and normal. Herein, our studies show that all IIP primary fibroblast lines constitutively express IL-4Rα, IL-13Rα1, and/or IL-13Rα2 at the gene and protein level. Given the enhanced expression of IL-4 and IL-13 receptor expression in IIP fibroblasts, we also examined the effects of a fusion protein comprised of IL-13 and a mutated form of *Pseudomonas* exotoxin (IL-13-PE38QQR or IL13-PE), which has been used extensively to target and kill IL-13R-expressing tumor[25] and inflammatory[26] cells. Other studies have shown that IL13-PE-induced cytotoxicity in tumor cells is mediated, in part, through apoptotic and nitric oxide pathways.[27] In the present study, the proliferative responses of primary lung fibroblast lines grown from SLBs from UIP, NSIP, and RBILD patient groups were significantly attenuated by the presence of IL13-PE, but this chimeric protein had a minimal effect on the proliferative responses of fibroblast lines grown from normal SLBs.

Materials and Methods

Patients

The Institutional Review Board at the University of Michigan Medical School approved this study. All patients underwent clinical evaluation, including chest radiography, lung function measurements, and thin-section computed tomography before fiberoptic bronchoscopy. In these patients, a suspicion of IIP was determined from a compilation of symptoms, physiological symptoms, and radiographical findings. None of the patients enrolled in the present study had undergone previous biopsy surgery or received therapy for IIP. SLBs were obtained via the Clinical Core associated with the IIP Specialized Center of Research at the University of Michigan Medical School from patients suspected of having UIP, NSIP, or RBILD between May 2000 and May 2002. SLBs were obtained from at least two lobes (normally on the left side) in all patients undergoing diagnostic SLB for IIP as previously described in detail.[28] Histologically normal lung was obtained from resected specimens in patients undergoing thoracic resection. Each biopsy was processed separately using sterile technique in a laminar flow hood and processed for the culture of primary fibroblast lines (see below). Two pathologists (T.V.C. and W.D.T.) who were unaware of any other clinical findings independently reviewed each SLB, and histological classification was based on previously published criteria for IIP.[28–30] Detailed descriptions of the techniques used in this study are provided below.

Isolation and Culture of Primary Pulmonary Fibroblast Lines

IIP and normal SLBs were finely minced and the dispersed tissue pieces were placed into 150-cm^2 cell culture flasks (Corning Inc., Corning, NY) containing Dulbecco's modified Eagle's medium (DMEM, BioWhittaker, Walkersville, MD) supplemented with 15% fetal bovine serum (DMEM-15, BioWhittaker), 1 mmol/L glutamine (BioWhittaker), 100 U/ml penicillin (BioWhittaker), 100 μg/ml streptomycin (BioWhittaker), and 0.25 μg amphotericin B (Fungizone; BioWhittaker). All primary lung cell lines were maintained in DMEM-15 at 37°C in a 5% CO_2 incubator and were serially passaged a total of five times to yield pure populations of lung fibroblasts as previously described in detail.[31–33] All primary fibroblast cell lines were used at passages 6 to 10 in the experiments outlined below and all of the experiments were preformed under comparable conditions. Regardless of the passage number, the transcript and protein expression of the various IL-4 and IL-13 receptor subunits was stable during culture expansion and passage of primary human fibroblasts. In the following experiments, we analyzed eight UIP, four NSIP, three RBILD, and six normal primary fibroblast lines.

Preparation of RNA and cDNA from Fibroblast Cell Lines

Purified fibroblast lines from each patient were added to 24-well tissue culture plates at a cell density of 1×10^5 cells/well. Twenty-four hours after plating, fibroblasts were exposed to fresh DMEM-15 or DMEM-15 to which 10 ng/ml of IL-4, IL-13, IFN-γ, IL-12, or IL-10 had been added. All cytokines were purchased from R&D Systems (Minneapolis, MN). After 24 hours, TRIzol reagent was added to each well for RNA isolation. Purified RNA from SLBs and the fibroblast cell lines was subsequently

reverse-transcribed into cDNA using a BRL reverse transcription kit and oligo (dT) 12-18 primers. The amplification buffer contained 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, and 2.5 mmol/L $MgCl_2$.

Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) Amplification

Isolated mRNA from pulmonary fibroblast lines was also analyzed by RT-PCR. Specific oligonucleotide primers were added (200 ng/sample) to the buffer, along with 1 μl of reverse-transcribed cDNA sample. The following human oligonucleotide primers (5′ to 3′ sequences) were used for RT-PCR analysis: IL-4 receptor α sense, TGCGTCTCCGACTACATGAG and IL-4 receptor α anti-sense, TGACTGCATAGGTGAGATG (387-bp product); IL-13 receptor α1 sense, AAGGAATACCAGTCCCGACA and IL-13 receptor α1 anti-sense, ACCAGGGAACCATGAAACAAG (457-bp product); IL-13 receptor α2 sense, GTGAAACATGGAAGACCATC and IL-13 receptor α2 anti-sense, GTGAAATAACTGGATCTGATAGGC(453-bp product); common γ-chain sense, TCACTTCTGGCTGTCAGTTG and common γ-chain anti-sense, GGCATCGTCCGTTCCAGCCA (534-bp product); procollagen type I sense, CACTGGTGCTAAGGGAGAGC and procollagen type I anti-sense, CTCCAGCCTCTCCATCTTTG (521-bp product); and procollagen type III sense, AAGAAGGCGGAGAAGGACTC and procollagen type III anti-sense, ATCTGCATGATGAGGCTGTC (401-bp product). Mixtures containing cDNA and IL-4Rα, IL-13Rα1, IL-13Rα2, common γ-chain (γ-c), procollagen type I or procollagen type III sense and anti-sense primers were then first incubated for 4 minutes at 94°C and amplified using the following cycling parameters: cycled 38 times at 94°C for 45 seconds, 60°C for 60 seconds, and elongated at 72°C for 60 seconds. After amplification the PCR products were separated on a 2% agarose gel containing 0.3 μg/ml of ethidium bromide and bands visualized using a Molecular Imager FX ProPlus (Bio-Rad, Hercules, CA). Digitized images of each gel were then analyzed using PDQuest Analysis software (Bio-Rad) and the ratio of the density of the receptor subunit to β-actin was then calculated.

Immunocytochemistry

Paraformaldehyde-fixed pulmonary fibroblasts were analyzed for immunohistochemical localization of IL-4Rα, IL-13Rα1, and IL-13Rα2. All antibodies directed against these receptor subunits were obtained from R&D Systems. Primary human fibroblasts were plated in eight-well Labtek tissue culture slides (at a density of 1×10^3 cells/well), and 24 hours later these cells were fixed with 4% paraformaldehyde. These cells were subsequently thoroughly washed and blocked with normal rabbit serum (Vectorstain ABC-AP kit; Vector Laboratories, Burlingame, CA). Goat anti-human IL-4Rα, IL-13Rα1, and IL-13Rα2 antibodies and control normal goat IgG, were diluted in phosphate buffered saline (PBS) to a final concentration of 2 μg/ml. Antibodies or IgG were added to Labtek wells for 30 minutes after which each tissue section was washed thoroughly three times with PBS. A secondary rabbit anti-goat biotinylated antibody (Vector Laboratories) was added to each section for 60 minutes, then each slide was thoroughly washed and to each was added Avidin DH and biotinylated alkaline phosphatase (Vector Laboratories). Receptor localization was revealed with the Vector Red substrate kit. Coverslips were applied to each slide using an aqueous mounting solution.

Fibroblast Proliferation Analysis

Human fibroblast proliferation was assessed in 24-well tissue culture plates via [^{3}H]thymidine incorporation. Primary IIP and normal fibroblast lines were plated at a density of either 1×10^5 or 5×10^4 cells/well, and allowed to adhere overnight. To quadruplet wells plated with 1×10^5 cells/well, increasing concentrations of IL13-PE (0, 10, 100, 200, or 1000 ng/ml of PBS) were added to each well for 24 hours. To quadruplet wells plated with 5×10^4 fibroblasts/well the following was added: DMEM-15 alone, DMEM-15 + 10 ng/ml of human IL-4, IL-13, IFN-γ, IL-12, or IL-10. These culture plates were exposed to DMEM-15 alone or DMEM-15 + cytokine for 24 hours before the addition of IL13-PE (at 200 ng/ml) or diluent (PBS-HSA) alone for an additional 24 hours.

To examine which IL-4 and IL-13 receptor subunits IL13-PE was binding on IIP fibroblasts, neutralization studies were conducted using specific antibodies directed against the individual IL-4 and IL-13 receptor subunits. A range of antibody concentrations were tested in pilot proliferation studies and the optimal concentration for individual receptor subunit neutralization were as follows: 20 μg/ml of monoclonal mouse anti-human γ-c, 20 ng/ml monoclonal mouse anti-human IL-4Rα, 30 μg/ml polyclonal goat anti-human IL-13Rα1, and 2 μg/ml of polyclonal goat anti-human IL-13Rα2 (all were obtained from R&D Systems). These concentrations of receptor subunit antibody abolished the proliferative effects of exogenously added IL-4 or IL-13 to cultures of fibroblasts, and closely matched the recommended neutralizing concentrations provided by R & D systems. Primary UIP, NSIP, and RBILD fibroblast lines were plated at a density of 5×10^4 fibroblasts/well, and the following neutralization antibody combinations were then added to quadruplet wells of serum-deprived fibroblasts: the appropriate concentration of control mouse or goat IgG (R&D Systems), monoclonal mouse anti-human γ-c, monoclonal mouse anti-human IL-4Rα, polyclonal goat anti-human IL-13Rα1, polyclonal goat anti-human IL-13Rα2, or various combinations of the appropriate control IgG or anti-receptor subunit antibodies. All tissue culture plates were incubated at 37°C in a 5% CO_2 incubator for the duration of each experiment. Four hours before the conclusion of each experiment, 10 μCi of [^{3}H] thymidine was added to each well. Subsequently, fibroblasts were then washed three times with PBS and to each well 1 ml of 1% Triton X-100 was added for ~30 minutes. The Triton X-100-treated fibroblast samples were then added to 5-ml scintillation vials, 3 ml of scin-

tillation fluid was added, and the vials were analyzed in a Beckman scintillation counter (model LS 5801; Beckman Instruments, Fullerton, CA).

Statistical Analysis

All results are expressed as mean ± SEM (SE). InStat 3.0a for OSX (Apple MacIntosh) was used to determine statistical differences. One-way analysis of variance and Student-Newman-Keuls multiple comparison post-test were used to reveal statistical differences between the control and IL13-PE treatment groups for each IIP and normal fibroblast line. Two-way analysis of variance and the Bonferroni post-test were used to detect statistical differences related to cytokine and/or IL13-PE treatment on each fibroblast line or between the fibroblast lines. A three-way analysis of variance was used to take into account for possible differences between the upper and lower lobe fibroblast responses to cytokine and/or IL13-PE treatment. $P < 0.05$ was considered statistically significant.

Results

IL-4Rα, IL-13Rα1, IL-13Rα2, Procollagen I, and Procollagen III Gene Expression in Primary Fibroblast Lines Derived from IIP and Normal SLBs

Representative RT-PCR analysis of mRNA from IIP and normal SLB-derived fibroblasts revealed a number of differences in the expression of IL-4 and IL-13 receptor subunits and the procollagens I and III. All primary fibroblast lines were left untreated (media group) or exposed to 10 ng/ml of IFN-γ or IL-13 for 24 hours before the isolation of RNA and RT-PCR analysis. As shown in Figure 1 (representative RT-PCR gel above and densitometry analysis of all primary IIP and non-IIP fibroblast lines analyzed below), all three receptor subunits and both procollagens were constitutively expressed at much greater levels in the IIP compared with the normal fibroblast lines. In all treatment groups, UIP and NSIP fibroblast lines expressed significantly higher transcript levels of IL-4Rα compared with the appropriately treated normal fibroblast group (Figure 1). No statistical differences were detected for IL-13Rα1 transcript expression between the IIP and normal fibroblast lines examined. However IL-13Rα2 transcript expression was significantly higher in untreated and cytokine-treated UIP and NSIP fibroblast lines compared with untreated and cytokine-treated normal fibroblasts (with the exception of IL-13-treated UIP fibroblasts). No statistically significant effects of cytokine treatment on IL-4 and IL-13 receptor subunit gene expression were noted in any of the groups of primary fibroblast lines at the 24-hour time point shown. Both procollagen I and III transcript levels were significantly elevated in primary cultures containing untreated or cytokine-treated UIP and RBILD fibroblasts compared with normal fibroblast lines (Figure 1). No statistically significant effects of cytokine treatment procollagen I and III transcript levels were noted in any of the groups of primary fibroblast lines at the 24-hour time point shown. No constitutive or cytokine-induced common γ-chain gene expression was detected in any of the primary human fibroblast lines examined in this experiment (data not shown). Thus, these data showed that IL-4 and IL-13 receptor subunits appeared to be differentially regulated at the transcript level in primary IIP and normal fibroblast lines.

IL-4Rα, IL-13Rα1, and IL-13Rα2 Protein Expression in Primary Fibroblast Lines Derived from IIP and Normal SLBs

Given the divergent gene expression for IL-4 and IL-13 receptor subunits, we next examined the protein expression of each subunit on representative fibroblasts lines from IIP and normal patients. Among the fibroblast lines examined, the UIP fibroblast lines ($n = 8$) exhibited the highest levels of IL-4 and IL-13 receptor subunits. For all fibroblast lines examined, IL-13Rα1 protein was constitutively present and its expression did not appear to be markedly affected by cytokine treatment (not shown). Representative staining for IL-4Rα and IL-13Rα2 in UIP and NSIP fibroblast lines are shown in Figures 2 and 3, respectively. Strong constitutive (Figure 2B) and IL-13-induced (Figure 2D) IL-4Rα protein expression was observed in UIP fibroblast lines. Exposure of these fibroblasts to IFN-γ markedly inhibited the expression of this IL-4 receptor subunit (Figure 2C). Constitutive IL-4Rα protein expression was weakly expressed in NSIP fibroblasts (Figure 2F), and its expression was inhibited by IFN-γ (Figure 2G) whereas IL-13 did not appear to induce IL-4Rα expression (Figure 2H). UIP fibroblasts exhibited constitutive IL-13Rα2 protein expression (Figure 3B). Greater expression of IL-13Rα2 was observed in cultures of UIP fibroblasts exposed to IFN-γ (Figure 3C) or IL-13 (Figure 3D). NSIP fibroblasts exhibited no constitutive (Figure 3F) or IFN-γ (Figure 3G)-induced expression for IL-13Rα2 protein. However, NSIP fibroblasts exhibited low IL-13Rα2 protein expression when exposed to IL-13 for 24 hours (Figure 3H). We also observed constitutive and inducible IL-4Rα and IL-13Rα2 protein expression in cultures of RBILD fibroblasts that was comparable to that observed in cultures of NSIP fibroblasts, but less than that observed in cultures of UIP fibroblasts (not shown). Conversely, primary normal fibroblast lines expressed very low constitutive and IL-13-inducible levels of all receptor subunits relative to levels observed in the IIP fibroblast lines (not shown). IFN-γ appeared to have no effect on the expression of either receptor subunit on primary RBILD and normal fibroblast lines. Thus, these findings show that receptor subunit protein expression varies considerably among the IIP and normal fibroblast lines examined herein.

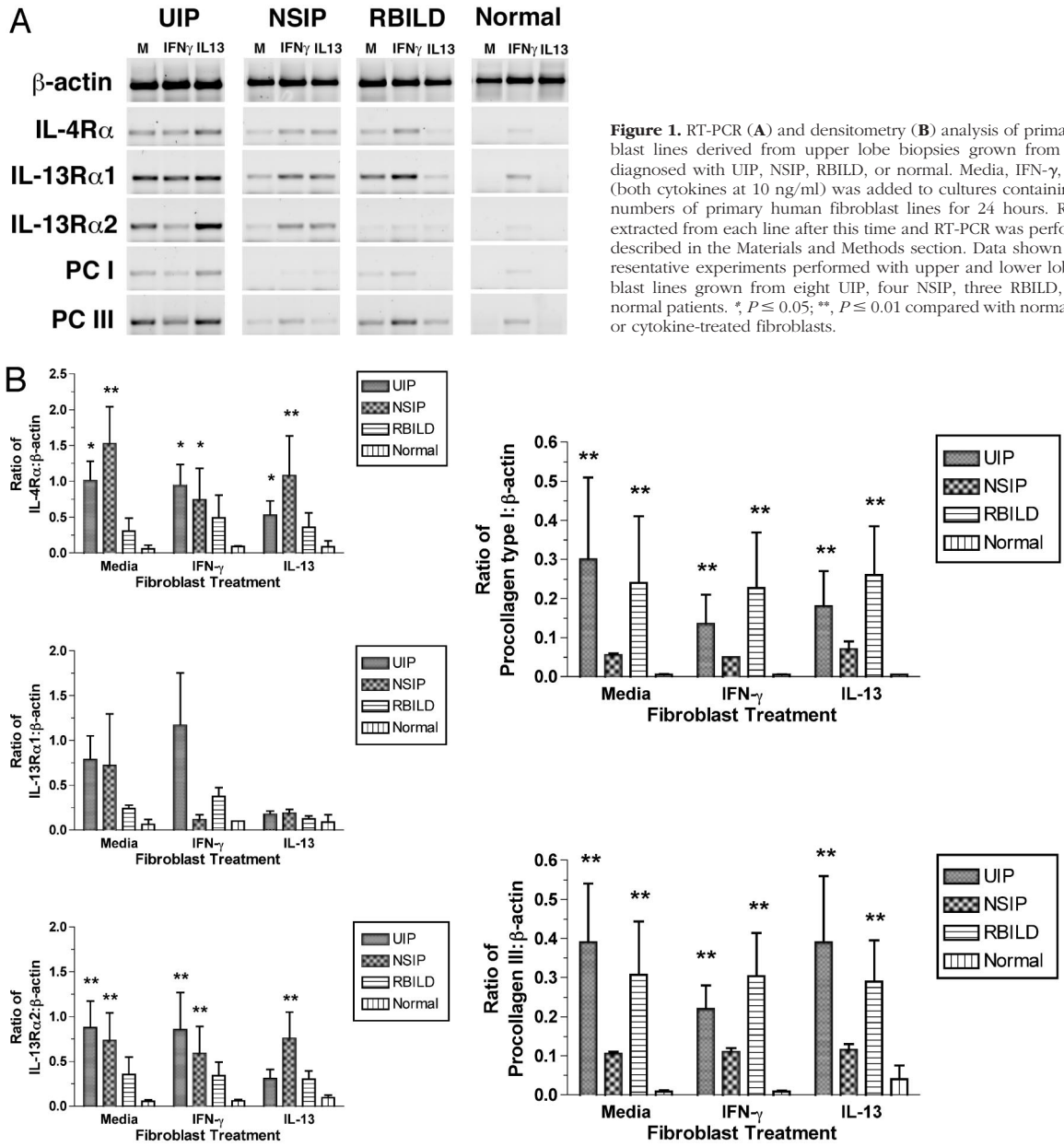

Figure 1. RT-PCR (**A**) and densitometry (**B**) analysis of primary fibroblast lines derived from upper lobe biopsies grown from patients diagnosed with UIP, NSIP, RBILD, or normal. Media, IFN-γ, or IL-13 (both cytokines at 10 ng/ml) was added to cultures containing equal numbers of primary human fibroblast lines for 24 hours. RNA was extracted from each line after this time and RT-PCR was performed as described in the Materials and Methods section. Data shown are representative experiments performed with upper and lower lobe fibroblast lines grown from eight UIP, four NSIP, three RBILD, and six normal patients. *, $P \leq 0.05$; **, $P \leq 0.01$ compared with normal media- or cytokine-treated fibroblasts.

Specific Targeting of IIP Fibroblast Lines with IL13-PE: Effects on Proliferative Properties

The immunohistochemical analysis of the IIP and normal fibroblast lines suggested that each cell line may exhibit differential sensitivity to the presence of IL13-PE. We have previously observed that IL13-PE significantly inhibits the proliferation of mouse fibroblasts derived from Th1-type and Th2-type granulomas, but this immunotoxin does not target normal mouse fibroblasts.[34] In the present study, compared with tissue culture wells containing UIP fibroblasts exposed to the vehicle for IL13-PE (Figure 4A, top), a dramatic reduction in the numbers of viable UIP fibroblasts was observed when 200 ng/ml of IL13-PE was added to identical tissue culture wells for 24 hours (Figure 4B, top). In contrast, tissue culture wells of NSIP fibroblasts exposed to the vehicle for IL13-PE (Figure 4C, top) and identical tissue culture wells of NSIP fibroblasts exposed to 200 ng/ml IL13-PE appeared to contain similar numbers of fibroblasts (Figure 4D, top). Consequently, the dose-dependent effect of IL13-PE on the proliferation of the human primary IIP and normal fibroblasts was examined and these data are summarized in Figure 4 (bottom). IL13-PE had the greatest dose-dependent inhibitory effect on the proliferation of UIP fibroblasts compared with all other fibroblast lines examined. In these experiments, the presence of 200 ng/ml of IL13-PE suspended in growth medium for 24 hours reduced [^{3}H]thymidine incorporation by ~65 ± 17% in cultures of UIP fibroblasts, by 24 ± 27% and 29 ± 23%, respectively, in cultures of NSIP and RBILD fibroblasts and by 16 ± 10% in cultures of normal fibroblasts. Greater inhibition of [^{3}H]thymidine incorporation was observed in all four fibroblast lines when the concentration

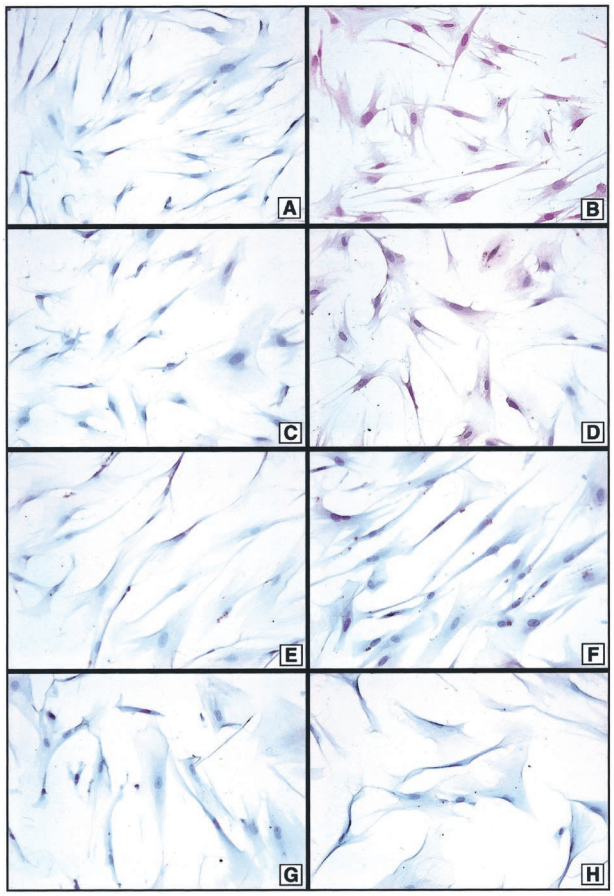

Figure 2. IL-4Rα protein expression in upper lobe UIP (**A–D**) and upper lobe NSIP (**E–H**) primary fibroblast lines. **A** and **E:** Negative control staining for the respective fibroblast line. Representative IL-4Rα staining (red) is shown for untreated fibroblasts (**B** and **F**), those exposed to IFN-γ (**C** and **G**) or IL-13 (**D** and **H**). The staining shown is representative of upper lobe fibroblast lines from the eight UIP and four NSIP patients examined in this study. Original magnifications, ×200.

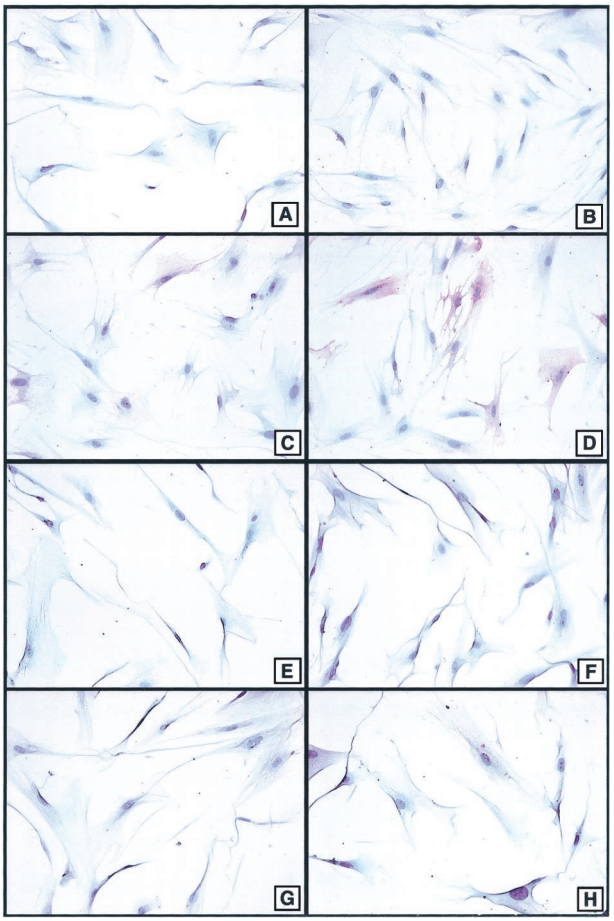

Figure 3. IL-13Rα2 protein expression in upper lobe UIP (**A–D**) and upper lobe NSIP (**E–H**) primary fibroblast lines. **A** and **E:** Negative control staining for the respective fibroblast line. Representative IL-13Rα2 staining (red) is shown for untreated fibroblasts (**B** and **F**), those exposed to IFN-γ (**C** and **G**) or IL-13 (**D** and **H**). The staining shown is representative of upper lobe fibroblast lines from the eight UIP and four NSIP patients examined in this study. Original magnifications, ×200.

of IL13-PE was increased to 1000 ng/ml (Figure 4, bottom). In cultures of UIP fibroblasts, the inhibition of fibroblast proliferation was 77 ± 10%, whereas proliferative responses in cultures of NSIP and RBILD fibroblasts were reduced by 35 ± 25% and 45 ± 15%, respectively (Figure 4, bottom). The presence of 1000 ng/ml of IL13-PE in cultures of normal fibroblasts had a minor effect on the proliferation of these cells because the proliferation of these cells was only reduced by 20 ± 11% (Figure 4, bottom). The growth inhibitory effect of both concentrations of IL13-PE on UIP fibroblasts was significantly greater than the growth inhibitory effect of either concentration of this chimeric protein on normal fibroblasts.

Cytokine-Pretreated IIP but Not Normal Fibroblast Lines Were Susceptible to the Anti-Proliferative Effects of IL13-PE

We next assessed whether IL-4, IFN-γ, IL-10, IL-12, or IL-13 treatment of IIP and non-IIP fibroblast lines affected the response by these primary fibroblast lines to the presence of IL13-PE (Figure 5). Surprisingly, [^{3}H]thymidine incorporation by UIP fibroblasts in control tissue culture wells was not altered by the presence of any of these cytokines, whereas all three other primary fibroblast lines exhibited altered proliferation profiles after cytokine treatment (ie, media *versus* cytokine treatment groups). In control cultures of RBILD fibroblasts, the presence of 10 ng/ml of IFN-γ, IL-10, or IL-12 significantly increased the [^{3}H]thymidine incorporation by these cells whereas IL-13 had the opposite effect. In control cultures of NSIP fibroblasts, IL-10 significantly increased the [^{3}H]thymidine incorporation by these cells. Conversely, in cultures of normal fibroblasts the presence of IL-4, IFN-γ, or IL-10 significantly decreased the [^{3}H]thymidine incorporation by these cells. In IL13-PE-treated tissue culture wells, few statistical differences in the [^{3}H]thymidine incorporation were detected between the media and cytokine treatment groups (Figure 5). After a 24-hour exposure to a number of Th1- and Th2-type cytokines, IL13-PE significantly inhibited the [^{3}H]thymidine incorporation by pulmonary fibroblasts grown, purified, and expanded from UIP and RBILD patients compared with the appropriate control wells containing fibroblasts from these two

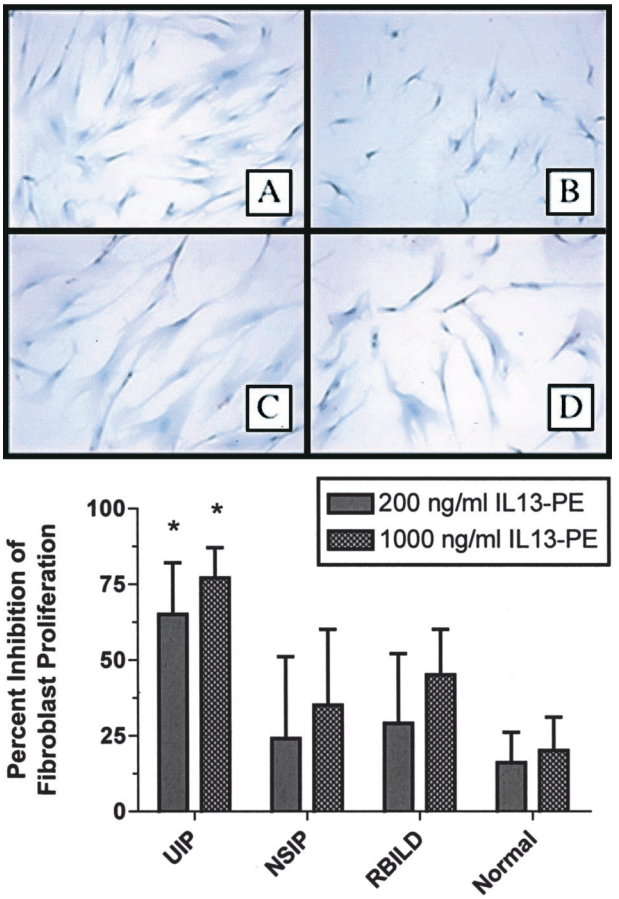

Figure 4. Effect of IL13-PE on fibroblast appearance (**top**) and fibroblast proliferation (**bottom**). Untreated UIP and NSIP fibroblasts are shown in **A** and **C**, respectively (**top**). **B:** The addition of 200 ng/ml of IL13-PE to cultures of UIP fibroblasts for 24 hours dramatically reduced the numbers of fibroblasts in these cultures, and the remaining fibroblasts appeared sickly. **D:** The addition of the same concentration of IL13-PE to cultures of NSIP fibroblasts for the same amount of time also reduced the numbers of fibroblasts but to a much smaller extent. In addition, many of the remaining fibroblasts exposed to IL13-PE were similar in appearance to those seen in untreated cultures (**C** *versus* **D**). Percent inhibition of fibroblast proliferation after IL13-PE treatment *in vitro*. Quantitative analysis of the anti-proliferative effect of IL13-PE on fibroblast lines from the four patient groups is shown at the **bottom**. Fibroblasts were left untreated or exposed to PBS-HSA (vehicle for IL13-PE), 200 or 1000 ng/ml of IL13-PE for 24 hours. The percent inhibition of fibroblast proliferation is indicated for each IL13-PE treatment group. Data shown are mean ± SE of the percent inhibition in fibroblast proliferation observed in quadruplicate vehicle-treated wells *versus* quadruplicate IL13-PE-treated wells. The data shown is from the following number of upper and lower lobe fibroblast lines: UIP ($n = 8$), NSIP ($n = 4$), RBILD ($n = 3$), and normal ($n = 6$). *, $P \leq 0.05$ compared with normal fibroblast groups exposed to either 200 or 1000 ng/ml of IL13-PE.

IIP patient groups (Figure 5). However, [^{3}H]thymidine incorporation was not inhibited in cultures of IL-13-stimulated UIP and RBILD fibroblasts by the presence of 200 ng/ml IL13-PE. NSIP fibroblasts exposed to media alone or media + IFN-γ exhibited significant decreases in [^{3}H]thymidine incorporation whereas no changes in the proliferation profile of normal fibroblasts was observed regardless of the fibroblast treatment (Figure 5). Thus, these data demonstrate that cytokine-treated UIP and RBILD, but not NSIP or normal, fibroblasts are highly susceptible to the anti-proliferative effects of IL13-PE *in vitro*.

UIP is a progressive fibrotic disease that exhibits lobar histological variability with lower lobe biopsies typically showing more pronounced evidence of fibrotic disease compared with upper lobe biopsies.[28] From each IIP patient, primary fibroblast lines were grown concomitantly from both upper and lower lobe biopsies. Similar baseline (M) proliferation was observed in both primary fibroblast lines (Figure 6). Similarly, cytokine treatment failed to alter the proliferation of upper lobe UIP fibroblasts but IL-13 treatment significantly increased the [^{3}H]thymidine incorporation by lower lobe UIP fibroblasts compared with UIP fibroblasts given media alone. In this experiment, primary UIP fibroblast lines from upper and lower lobe biopsies were exposed to Th1 and Th2 cytokines for 24 hours before the addition of 200 ng/ml of IL13-PE. Three-way analysis of variance statistical analysis revealed that enhanced susceptibility to the targeting effects of IL13-PE was observed in primary fibroblasts derived from lower lobe UIP biopsies *versus* upper lobe biopsies, specifically in cultures of lower lobe fibroblasts exposed to 10 ng/ml of IFN-γ, IL-10, IL-12, or IL-13 (Figure 6). The proliferation of UIP fibroblasts derived from the lower lobe biopsy was reduced by greater than 60% in all culture conditions exposed to IL13-PE, whereas the upper lobe biopsy line was much less susceptible to IL13-PE and the presence of exogenous IL-13 blocked its effects in this fibroblast line. Thus, these data show that UIP fibroblasts, particularly those grown from lower lobe biopsies, where interstitial fibrosis is usually more severe, are susceptible to the targeting effects of IL13-PE.

Receptor Subunit Specificity of IL13-PE: IL-4Rα and IL-13Rα2 Subunits Are Key to UIP Fibroblast Susceptibility to IL13-PE

To determine which receptor subunit(s) were necessary for the anti-proliferative or targeting effects of IL13-PE, the next series of fibroblast proliferation experiments included specific antibodies directed against each IL-4 and IL-13 receptor subunit alone or in combination. Representative results are shown in Figures 7 and 8. The inclusion of antibodies directed against either the IL-13Rα1 subunit or common γ-c alone to cultures of UIP, NSIP, or RBILD fibroblasts did not reverse the anti-proliferative effects of IL13-PE (not shown). An antibody directed against the IL-4Rα subunit alone had no effect on the targeting effects of IL13-PE in cultures of UIP fibroblasts but this antibody reduced the anti-proliferative effects of IL13-PE in cultures of NSIP and RBILD fibroblasts (Figure 7). However, the [^{3}H]thymidine incorporation in cultures containing UIP, NSIP, and RBILD fibroblasts to which anti-IL-4Rα and IL13-PE were added was significantly greater than [^{3}H]thymidine incorporation in identical cultures of primary fibroblast lines to which control mouse IgG2a and IL13-PE were added. Anti-IL-13Rα2 alone failed to alter the anti-proliferative effect of IL13-PE on UIP and RBILD fibroblasts whereas this antibody reduced the anti-proliferative of IL13-PE in cultures of NSIP fibroblasts (Figure 7).

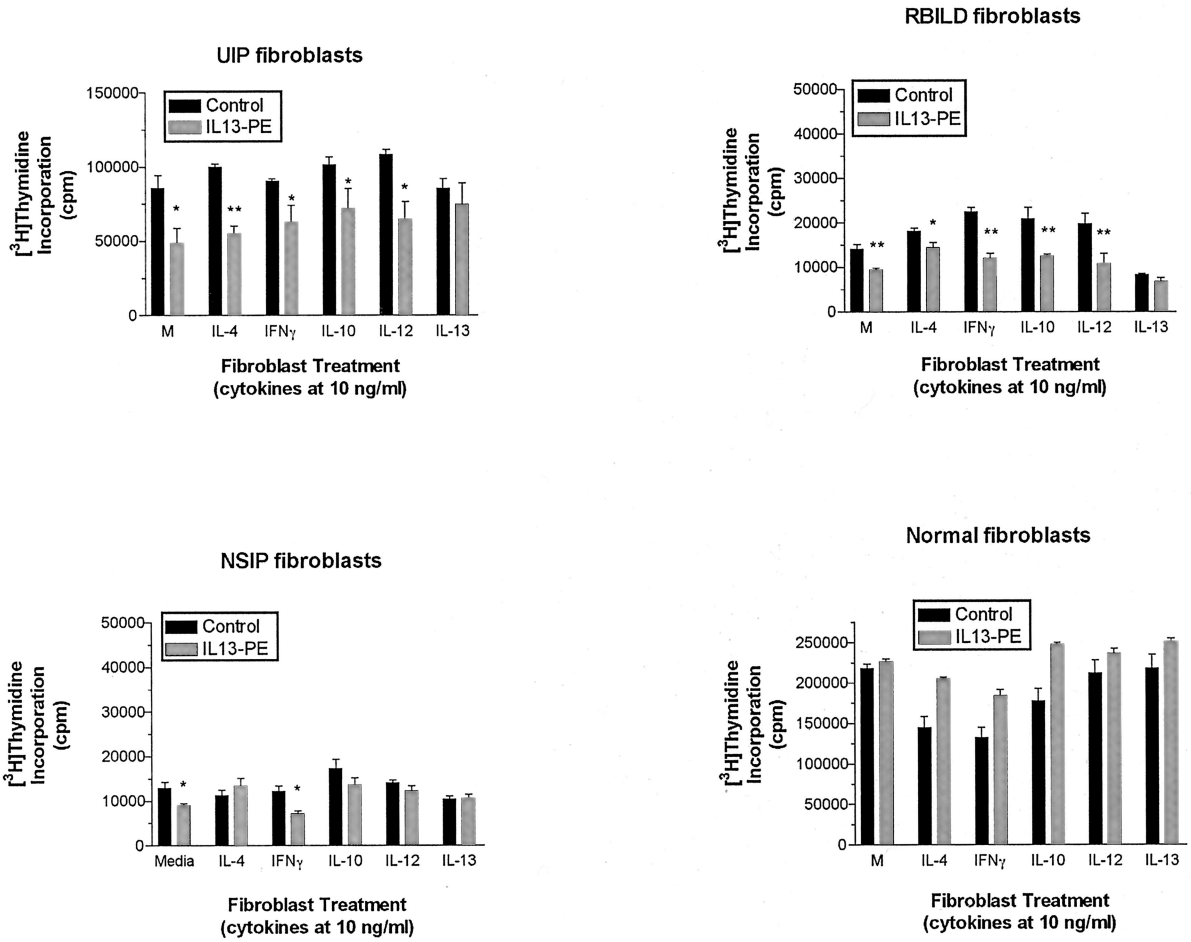

Figure 5. Quantitative analysis of the anti-proliferative effect of IL13-PE on four primary human fibroblast lines grown from upper lobe biopsies. Fibroblasts were left untreated (M) or exposed to 10 ng/ml of IL-4, IFN-γ, IL-10, IL-12, or IL-13 for 24 hours. Subsequently, either PBS-HSA (vehicle for IL13-PE, control group) or 200 ng/ml of IL13-PE was added to the fibroblast lines for an additional 24 hours. During the last 4 hours of the experiment, the fibroblast cultures were pulsed with [^{3}H]thymidine and the end of which the cells were harvested and analyzed in a scintillation counter. Except in cultures of fibroblasts exposed to IL-13, IL13-PE had a significant inhibitory effect on untreated and cytokine-activated UIP and RBILD fibroblast proliferation. In contrast, IL13-PE had a negligible inhibitory effect on NSIP fibroblast proliferation; only untreated and IFN-γ-treated NSIP fibroblasts showed a significant decrease in fibroblast proliferation after IL13-PE. IL13-PE did not inhibit constitutive or cytokine-altered proliferation in cultures of normal fibroblasts. Data shown are mean ± SE of quadruplicate wells and are representative of three separate experiments. Similar results were obtained from all eight UIP, four NSIP, three RBILD, and six normal primary upper lobe fibroblast lines examined in this study. $\tau \leq 0.05$, $\tau\tau \leq 0.01$, $\tau\tau\tau$ 0.001 compared with control- or IL13-PE-treated fibroblasts to which media (M) alone was added. *, $P \leq 0.05$; **, $P \leq 0.01$ compared with media- or cytokine-treated fibroblasts to which PBS-HSA (control group) was added.

When antibodies were added in combination to cultures of IIP fibroblasts, only the dual inclusion of antibodies directed against IL-4Rα and IL-13Rα2 completely reversed the anti-proliferative effects of IL13-PE (Figure 8). Interestingly, the dual inclusion of antibodies directed against IL-4Rα and IL-13Rα2 significantly decreased the [^{3}H]thymidine incorporation in control (PBS-HSA) cultures of UIP fibroblasts compared with cultures of UIP fibroblasts to which control antibodies and PBS-HSA were added (Figure 8). This anti-proliferative effect was not observed in cultures of NSIP or RBILD fibroblasts. In all IIP fibroblast lines tested in the present study, the dual inclusion of antibodies directed against IL-4Rα and IL-13Rα2 with IL13-PE significantly increased the [^{3}H]thymidine incorporation in cultures of UIP, NSIP, and RBILD fibroblasts compared with cultures of the same fibroblast lines to which control antibodies and IL13-PE were added. Other antibody combinations, including anti-IL-13Rα1 + anti-γ-c, anti-IL-13Rα1+ anti-IL-13Rα2, or anti-IL-13Rα2 + anti-γ-c did not reverse the fibroblast targeting effects of IL13-PE whereas, antibody combinations of anti-IL-13Rα1 + anti-IL-4Rα and anti-IL-4Rα + anti-γ-c demonstrated same levels of neutralization effectiveness as neutralization with IL-4Rα alone (not shown). Thus, these data suggested that IL-4Rα and IL-13Rα2 subunit expression were necessary for the susceptibility of IIP fibroblasts to the cytotoxic effects of IL13-PE.

Discussion

The most common histological pattern in IIP is idiopathic pulmonary fibrosis or UIP, which is associated with a median survival of less than 3 years.[35] Although the precipitating event(s) in this disease and other IIPs remain unknown, pathological processes including epithelial injury and activation, the formation of distinctive subepithelial fibroblast/myofibroblast foci, and excessive

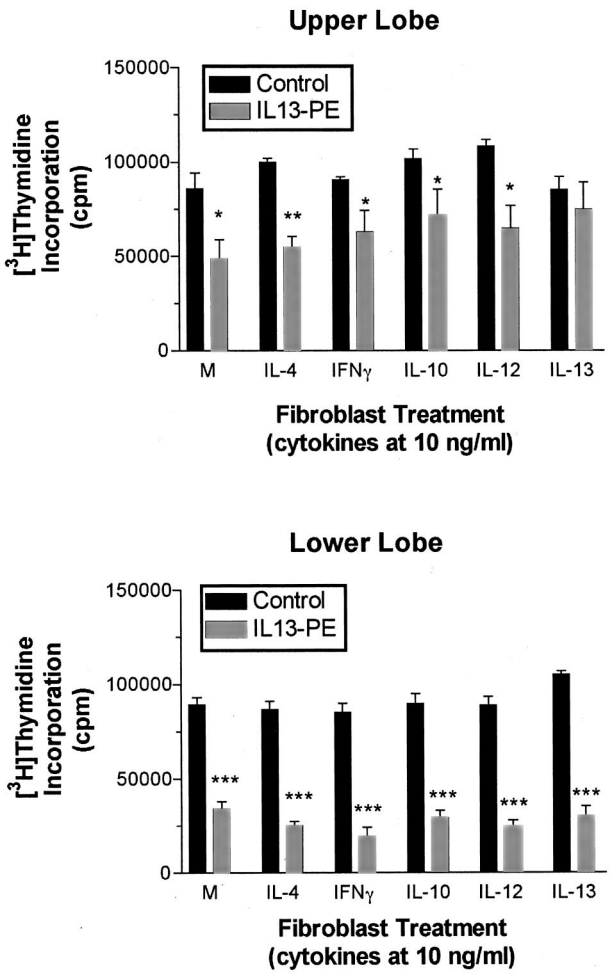

Figure 6. Quantitative analysis of the anti-proliferative effect of IL13-PE on primary human fibroblast lines derived from upper and lower lobe biopsies from an UIP patient. Fibroblasts were left untreated (M) or exposed to 10 ng/ml of IL-4, IFN-γ, IL-10, IL-12, or IL-13 for 24 hours. Subsequently, either PBS-HSA (vehicle for IL13-PE, control group) or 200 ng/ml of IL13-PE was added to the fibroblast lines for an additional 24 hours. During the last 4 hours of the experiment, the fibroblast cultures were pulsed with [^{3}H]thymidine and the end of which the cells were harvested and analyzed in a scintillation counter. Except in cultures of upper lobe fibroblasts exposed to IL-13, IL13-PE had a significant inhibitory effect on untreated and cytokine-activated UIP fibroblast proliferation. Data shown are mean ± SE of quadruplicate wells and are representative of three separate experiments. Similar results were obtained from all eight UIP primary upper and lower lobe fibroblast lines examined in this study. $\tau \leq 0.05$ compared with control- or IL13-PE-treated fibroblasts to which media (M) alone was added. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ compared with media- or cytokine-treated fibroblasts to which PBS-HSA (control group) was added.

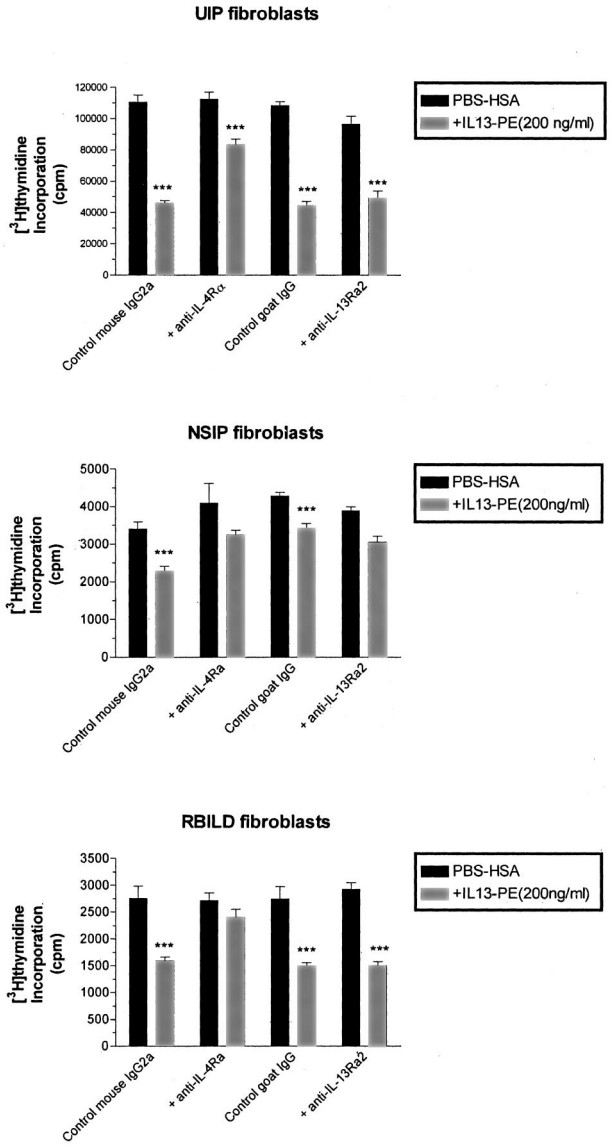

Figure 7. Quantitative analysis of the IL-4 and IL-13 receptor subunit involvement in the anti-proliferative effect of IL13-PE on upper lobe UIP, NSIP, and RBILD fibroblasts. Proliferation responses by all three fibroblast types were monitored after exposure to either PBS-HSA (vehicle for IL13-PE) or 200 ng/ml of IL13-PE in the presence of anti-IL-4Rα, anti-IL-13Rα2, or the appropriate control IgG antibodies for 24 hours. During the last 4 hours of each experiment, the fibroblast cultures were pulsed with [^{3}H]thymidine and at the end of which the cells were harvested and analyzed in a scintillation counter. The presence of anti-IL-4Rα antibody alone partially reversed (in UIP fibroblast cultures) and completely reversed (in NSIP and RBILD fibroblast cultures). Anti-IL-13Rα2 antibody treatment also reversed the anti-proliferative effect of IL13-PE in cultures of NSIP fibroblasts. Data shown are mean ± SE of quadruplicate wells. Similar results were obtained from all eight UIP, four NSIP, and three RBILD primary upper lobe fibroblast lines examined in this study. $\tau\tau\tau \leq 0.001$ compared with control- or IL13-PE-treated fibroblasts to which control antibody (ie, IgG2a or IgG) was added. ***, $P \leq 0.001$ compared with media- or cytokine-treated fibroblasts to which PBS-HSA was added.

extracellular matrix accumulation lead to progressive and irreversible changes in the lung architecture. Debate persists concerning the relative contribution of inflammatory mechanisms in these events primarily because of the ineffectiveness of many anti-inflammatory or immunomodulatory treatments on prolonging the survival of patients with this disease, but it is apparent that structural cells such as epithelial cells and fibroblasts exhibit abnormal synthetic and proliferative properties.[36] However, the large array of soluble mediators present in the fibrotic lung and the complexity of the networks they participate in have hampered the identification of the principle factors that are responsible for activating these cells to the point where they participate in a tissue disruptive wound-healing response.[2,37] An alternative therapeutic approach to this problem involves the specific targeting of these rogue cells within the lung. In this regard, it was growing evidence that Th2-type cytokines such as IL-4 and IL-13 are profibrotic cytokines that directed our at-

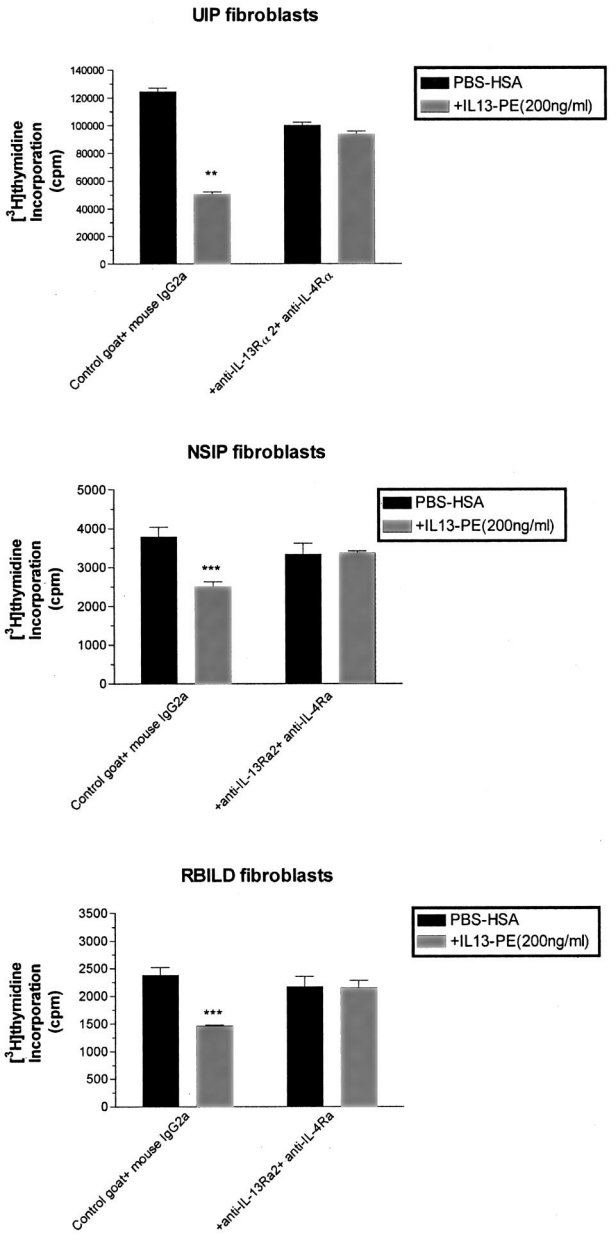

Figure 8. Quantitative analysis of the IL-4 and IL-13 receptor subunit involvement in the anti-proliferative effect of IL13-PE on UIP, NSIP, and RBILD fibroblasts. Proliferation responses by all three fibroblast types were monitored after exposure to either PBS-HSA (vehicle for IL13-PE) or 200 ng/ml of IL13-PE in the presence of combinations of anti-IL-4Rα, anti-IL-13Rα2, or appropriate control IgG antibodies for 24 hours. During the last 4 hours of each experiment, the fibroblast cultures were pulsed with [^{3}H]thymidine and at the end of which the cells were harvested and analyzed in a scintillation β-counter. The combination treatment of anti-IL-4Rα and anti-IL-13Rα2 antibodies completely reversed the anti-proliferative effect of IL13-PE on all three IIP fibroblast subtypes. Data shown are mean ± SE of quadruplicate wells. Similar results were obtained from all eight UIP, four NSIP, and three RBILD primary upper lobe fibroblast lines examined in this study. $\tau\tau\tau \leq 0.001$ compared with control- or IL13-PE-treated fibroblasts to which control (ie, IgG2a + IgG) antibodies were added. **, $P \leq 0.01$; ***, $P \leq 0.001$ compared with media- or cytokine-treated fibroblasts to which PBS-HSA was added.

tention to examining the expression of IL-4 and IL-13 receptor subunits on primary human fibroblasts from IIP SLBs and determining whether IIP fibroblasts could be selectively targeted with an IL-13-immunotoxin, which has been used extensively to target IL-13 receptor-positive tumor cells.[25,38]

The major functional IL-4/IL-13 receptor is comprised of the IL-4Rα and IL-13Rα1 subunits,[22,39,40] explaining, in part, why IL-4 and IL-13 have similar effects on pulmonary fibroblasts,[19,21,41,42] and similarly activate STAT-6/JAK1[24] and JAK2 tyrosine kinase-dependent signal transduction pathways.[16,21] Pulmonary fibroblasts also express the IL-13Rα2 subunit, a high-affinity decoy IL-13 receptor[20,22] with no signaling activity[43] that has ~100-fold higher affinity for IL-13 than IL-13Rα1.[44,45] In the present study, we observed differential expression for IL-4 and IL-13 receptor subunit at the gene and protein levels in UIP, NSIP, RBILD, and normal fibroblasts. These data are consistent with those of Doucet and colleagues[19] who demonstrated that human lung fibroblasts (either from fetal or adult tissues) express functional but different IL-4 and IL-13 receptors. Their study was the first to demonstrate in human fibroblasts that these cells could behave as inflammatory cells through the elaboration of proinflammatory cytokine and chemokine release via IL-4 or IL-13 activation.[19] One novel observation from the present study was that UIP fibroblasts appeared to express the highest levels of IL-4Rα and IL-13Rα2 compared with the other IIP and normal fibroblast lines, suggesting a disease-dependent effect on IL-4 and IL-13 receptor expression by pulmonary fibroblasts. It was also observed that exposure of primary human fibroblasts to IFN-γ or IL-13 had diverse effects on the expression of IL-4 and IL-13 receptor subunits that appeared to be disease-specific. For example, IFN-γ markedly decreased protein levels of the IL-4Rα subunit on UIP fibroblasts. In this regard, IFN-γ may have modulated protein translation and/or receptor turnover in primary UIP fibroblast lines because this Th1 cytokine did not modulate transcript levels of IL-4Rα. In addition, a marked stimulatory effect of IL-13 on IL-13Rα2 protein expression by UIP fibroblasts, which is consistent with previous findings by Yoshikawa and colleagues[46] who found that Th2 cytokines and TNF-α up-regulate the expression of IL-13Rα2 on human fibroblasts through induction of transcript expression and the mobilization of intracellular stores containing this receptor subunit. However, it was also observed that IFN-γ did not have the same effect on UIP fibroblasts, which contrasts with the published findings of Daines and Hershey.[47] In their studies it was noted that IL-13Rα2 was rapidly mobilized from intracellular stores in human U937, nasal epithelial cells, and monocytes after treatment with IFN-γ, and the up-regulation of IL-13Rα2 resulted in diminished IL-13 signaling in all three cell types.[47] Primary fibroblast lines were not examined in this previous study and thus it conceivable that fibroblasts do not respond to IFN-γ in the same manner, or that IIP fibroblasts have lost the ability to up-regulate IL-13Rα2 in response to this cytokine. Further studies are certainly warranted to address this dilemma.

Fibroblast proliferation and excess matrix deposition are two major attributes of IIP. However, controversy persists as to whether IIP fibroblasts proliferate faster than fibroblasts taken from normal lung.[48] The previous studies by Ramos and colleagues[48] carefully demon-

strated that primary fibroblast lines grown from SLBs from IIP patients grew at a slower rate and underwent spontaneous apoptosis compared with normal fibroblasts. Our findings concur with this previous study in that normal fibroblasts were found to incorporate greater amounts of [^{3}H]thymidine throughout a given time frame compared with IIP fibroblasts. In addition, we observed that, aside from the exogenous addition of 10 ng/ml of IL-13 to lower lobe fibroblast lines, the exogenous addition of diverse cytokines to cultures of UIP fibroblasts did alter the proliferative rate of these cells. In contrast, a number of cytokines either increased or decreased the overall [^{3}H]thymidine incorporation by NSIP, RBILD, and normal fibroblast lines. Although we did not examine this possibility, increased apoptosis by IIP fibroblasts may have accounted for their diminished responsiveness to exogenous cytokines and their overall proliferative rate. However, we consistently observed that regardless of the baseline proliferative rate of the fibroblast line, IIP fibroblast lines were targeted by IL13-PE whereas normal fibroblasts were primarily impervious to this chimeric cytotoxin. Again, the differential responsiveness appeared to be related to the relative protein expression of IL-4 and IL-13 receptor subunits.

Conflicting data are present in the literature pertaining to the expression of the common γ-c by fibroblasts[19,21,49] which may reflect the fact that normal fibroblasts do not express this chain whereas activated fibroblasts (ie, α-smooth muscle actin-positive fibroblasts or myofibroblasts) do.[24] Most recently, Doucet and colleagues[49] showed that activated lung fibroblasts or myofibroblasts express the common γ-c, but in the present study we failed to observe constitutive or cytokine-induced gene and protein expression of this receptor subunit in the IIP and normal fibroblast lines examined herein. In addition, a neutralizing antibody directed against the γ-c did not alter the IL13-PE-mediated targeting of IIP fibroblasts, nor did its addition to fibroblast cultures treated with anti-IL-4Rα antibody potentiate the reversing effects of this antibody. An explanation for the difference between our results and those of Doucet and colleagues[49] is not presently forthcoming but the expression of γ-c may reflect the culture conditions and/or the form of lung disease from which the fibroblasts are derived. The fibroblasts examined previously appeared to be derived from the airways of asthmatics whereas those in the present study were derived from interstitial areas of IIP and normal patients.

IL13-PE has been found to only target cells that express high numbers of IL-13 receptors (ie, $>$ 300 sites/cell) thereby excluding many bone marrow-derived cells such as T, B, and monocytic cells.[38] Previous studies have also shown that the cell-killing activity of IL13-PE was competed away by an excess of IL-13.[38] Increasing the sensitivity of cancer cells to the effects of IL13-PE was achieved by altering IL-13Rα2 expression on these cells.[50] IL-13Rα2 undergoes internalization after binding IL-13 but this response does not induce a signaling cascade, a property that is ideal in the context of IL13-PE-mediated targeting of IIP fibroblasts.[43] The molecular mechanism through which IL13-PE exerts its cytotoxic effect has been previously studied and it has been shown that this chimeric protein promotes apoptotic cell death in tumor cells.[51] In the present study, we observed that immunoneutralization of IL-13Rα2 alone did not block the targeting effects of IL13-PE. Instead, the immunoneutralization of IL-4Rα alone had the greatest effect in reversing IL13-PE's effects in cultures of IIP fibroblasts. Two explanations may account for the important role for IL-4Rα in mediating IL13-PE-induced cytotoxicity in IIP fibroblasts. First, the presence of IL-4Rα is necessary for the expression of IL-13Rα2 (C.J. and C.M.H, unpublished observations).[52] The inclusion of anti-IL-4Rα antibody to IIP fibroblast cultures may have reduced the cell surface expression of IL-13Rα2, thereby diminishing the cytotoxic activity of IL13-PE on these cells. Support for this hypothesis is observed in cultures of IIP fibroblasts treated with anti-IL-4Rα and anti-IL-13Rα2 antibodies in which the cytotoxic effects of IL13-PE were completely reversed. Second, the anti-IL-4Rα antibody treatment may have altered the ability of the IL-4Rα and IL-13Rα2 receptor subunits to interact with one another thereby diminishing the internalization of IL13-PE into IIP fibroblasts. Previous studies by Rahaman and colleagues[53] showed that the IL-13Rα2 binds to the cytoplasmic domain of IL-4Rα and acts as an inhibitor of IL-4-dependent signal transduction pathways in glioblastoma cells. Thus, the targeting of IIP fibroblasts by IL13-PE requires the presence of IL-4Rα and IL-13Rα2 receptor subunits.

In summary, the data presented in this study show that primary pulmonary human IIP fibroblasts can be targeted in a manner that reflects their expression of IL-4 and IL-13 receptor subunits. IL13-PE effectively targeted and killed fibroblasts from IIP patients (particularly fibroblasts from UIP patients), but this immunotoxin had a minimal effect on the proliferation of normal fibroblast lines, which either lacked or expressed very low amounts of IL-4 and IL-13 receptor subunits. Given that the extent of the cellularity in the alveolar space and the fibrosis in the alveolar wall had no effect on patient survival whereas the presence of granulation/connective tissue deposition or fibroblastic foci did,[54] we hypothesize that targeting the proliferative response of the pulmonary fibroblast is a therapeutic approach worthy of exploration in the treatment of interstitial fibrotic diseases for which treatment options are currently limited.

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