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Expression of CD154 (CD40 Ligand) by Human Lung Fibroblasts: Differential Regulation by IFN-γ and IL-13, and Implications for Fibrosis

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The CD40-CD40 ligand (CD40L) system (CD154) is a central means of immune cell communication crucial for Ig class switching and enhanced Ag presentation. CD40 is also a key signaling conduit to activate nonhematopoietic cells, such as fibroblasts and endothelial cells, to produce proinflammatory mediators. Disruption of the CD40-CD40L pathway reduces lung inflammation and fibrosis, autoimmune disease and atherosclerosis. Non-bone marrow-derived structural cells are not known to express CD40L. In this study, we reveal the intriguing finding that primary strains of human lung fibroblasts derived from normal and scarred lung express both CD40L mRNA and protein. Interestingly, CD40L expression is down-regulated by IFN-γ, a type 1 cytokine with antiscarring properties, and is up-regulated by the profibrogenic type 2 cytokine IL-13. Flow cytometry and laser confocal microscopy revealed that the majority of CD40L was located intracellularly. Importantly, fibroblast strains from human idiopathic pulmonary fibrosis tissue expressed increased levels of CD40L compared with fibroblasts from nonscarred lung. Fibroblasts in the scarred areas of human lung tissue expressed high levels of CD40L. Finally, the blood and lung lavage levels of CD40L are significantly elevated in fibrosis patients compared with normals. These new findings demonstrate that fibroblasts are a new source of CD40L and that those involved in scarring may have undergone a selected expansion for high CD40L expression. Moreover, the antifibrotic activity of IFN-γ may involve the down-regulation of fibroblast CD40L levels. We speculate that fibroblast-derived CD40L plays a role in promoting fibroblast activation and possibly in interaction with CD40 bearing cells. The Journal of Immunology, 2004, 172: 1862–1871.

The CD154 (CD40 ligand (CD40L)) protein is a 33 kDa type II membrane glycoprotein that was first described as a surface marker exclusive to activated CD4 T cells (1–4). It has since been shown to be expressed by other cell types including dendritic cells (5), B cells (6, 7), and most recently platelets (8). CD154 belongs to the TNF family of cytokines. In addition to the 33 kDa form of the protein, 31 kDa and/or 18 kDa versions of CD154 have been identified (9–11). These smaller versions of CD154 have been proposed to be secreted/shed from cells and may possess bona fide cytokine activity (9–11).

CD40, the receptor for CD154, was originally identified and functionally characterized as a B cell surface marker (12, 13). CD40 is a 45–50 kDa type I integral membrane glycoprotein and a member of the TNF receptor superfamily. CD40 is known to be a potent activation molecule for a variety of cell types. Cells known to express CD40 include professional APCs such as B lymphocytes (13–15) and dendritic cells (14, 16), as well as non-bone-marrow derived cells including fibroblasts (17–19), endothelial cells (20, 21), and keratinocytes (22, 23).

CD40-CD154 interactions were first described between B and T lymphocytes in the context of generating humoral immunity (4). Multimerization of surface CD40 by trimeric CD154 is believed to be an important first step in initiating the signaling cascade. Cross-linking by CD154 brings in proximity the cytoplasmic domains of CD40. This makes available binding sites for TNFR-associated factors (TRAFs) (24). Because the short cytoplasmic domain of CD40 has no intrinsic enzyme activity, binding to TRAFs is important in activating downstream signaling (24–26). The extent of TRAF binding may be related to the degree of CD40 cross-linking. TRAFs not only interact with CD40 but with other members of the TNFR superfamily (24–26). Competition for TRAF binding by CD40 and other TNFR family members may further fine-tune the activation process.

Fibroblasts have been shown to express functional CD40. Fibroblasts from a variety of human tissues respond to CD40 ligation by transducing signals that activate the nuclear translocation of the NF-κB transcription factor (18, 19). This causes the fibroblasts to synthesize high levels of the proinflammatory cytokines IL-6 and IL-8 (18, 19), induces hyaluronan synthesis (27), expression of cyclooxygenase-2 and its downstream product PGE2 (28), and also increases their expression of the adhesion molecules ICAM-1 and VCAM-1 (29). Fibroblast expression of these molecules thus amplifies inflammatory and repair cascades.

Fibroblasts are a heterogeneous group of dynamic cells. They provide structural integrity to a tissue by synthesizing the supporting network of the extracellular matrix (ECM). Although, fibroblasts were originally thought of as mere structural cells, it is now

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3 Abbreviations used in this paper: CD40L, CD40 ligand; ECM, extracellular matrix; TRAF, TNFR-associated factor; UIP, usual interstitial pneumonia; IPF, idiopathic pulmonary fibrosis; B/AL, bronchial/alveolar lavage; NLF, normal lung fibroblast; PFL, fibrotic lung fibroblast.
clear that they take a much more active role in both the inflammatory and wound healing responses. Fibroblasts have been described as sentinel cells of the immune system; they define a tissue’s microenvironment by synthesizing chemokines, cytokines, and ECM, and participate in direct cross-talk with lymphocytes via the CD40-CD154 pathway (30–32).

Fibroblasts hold a central role in the normal wound healing process and in the development of fibrosis (scarring) in the lung, skin, and other tissues. Wound healing is a complex process of highly ordered events that normally results in restoration of tissue with minimal loss of function. It is divided into three overlapping phases: inflammation, remodeling, and resolution (33). Fibrosis occurs as a result of progressive and uncontrolled wound healing (33, 34). In the case of pulmonary fibrosis, fibroblast hyperplasia in concert with the deposition of excessive amounts of ECM components thickens and destroys alveolar capillary septae and leads to compromised ability for gas exchange (33–35). Development of fibrosis results from disordered and uncontrolled repair. Lung fibrosis is characteristic of many diseases including rheumatoid arthritis, scleroderma, idiopathic pulmonary fibrosis (IPF), and sarcoidosis and occurs as a consequence of treatment for cancer (e.g., ionizing radiation) and exposure to inhaled toxicants (33, 34).

Fibroblast activation is important for both normal wound healing and the development of fibrosis. Fibroblasts activated by inflammatory stimuli are responsible for the remodeling phase of the healing process. They migrate into the wound along the fibronectin matrix, begin to proliferate, and lay down ECM components, especially collagens (33). Dysfunction at any stage of this process may be the underlying basis for many scarring disorders of the lung and other organs. Whereas under normal conditions fibroblasts ultimately curb their ECM production, those in a profibrotic environment continue to proliferate and deposit matrix components. There is now ample evidence that fibroblasts play a central role in diseases in which there is persistent inflammation, and that fibroblasts derived from the diseased tissue have an altered pattern of cytokine, chemokine, and ECM synthesis compared with fibroblasts taken from normal tissue of the same anatomical origin (36–38).

Although fibroblast activation is important for the pathological development of fibrosis, the factors that contribute to this activation are not well understood. The CD40-CD154 pathway potently activates human lung fibroblasts in vitro (18, 27, 31). Activation of lung fibroblasts in vivo is likely to be a result of the signaling cascade initiated by CD40 ligation, as well as the context (cytokine microenvironment) in which these signals are received. Mouse models of lung injury support the concept that the CD40-CD154 pathway plays a critical role in the development of injury in vivo. For example, both ionizing radiation and oxygen-induced lung damage are substantially blocked in mice that received anti-CD154 Ab (39, 40). Another example of the importance of the CD40-CD154 pathway in the lung was established by Wiley et al. (41) who found that intranasal administration of soluble CD154 elicited pulmonary inflammation in wild-type but not CD40 knockout mice.

Because fibroblasts have been shown to express functional CD40, it has been proposed that they interact with infiltrating CD154+ T cells resulting in fibroblast activation. However, T cell expression of CD154 is transient, with rapid down-regulation upon binding to CD40 (42). In contrast, fibrosis is a chronic and progressive disease that probably requires ongoing signals for fibroblast activation. This led us to look for an alternative source of CD154 in the lung. In this study, we demonstrate for the first time that human lung fibroblasts are themselves a novel source of CD154 and propose that dysregulated expression of CD154 by resident fibroblasts could result in their chronic activation leading to lung fibrosis.

Materials and Methods

Fibroblast strains and cell culture

Scared human lung tissue was obtained from open lung biopsies performed for the diagnosis of the usual interstitial pneumonia (UIP) form of IPF or fibrotic pulmonary sarcoidosis. Unscared control tissue was obtained from patients undergoing surgical resections for nonmalignant nodules. Control tissue was selected furthest from the nodule and was histologically normal. Ethical permission for lung tissue collection was obtained from the University of Rochester Research Subjects Review Board (Rochester, NY). Primary strains of human lung fibroblasts were isolated as previously described (17). The fibroblasts were maintained in MEM with Eagle’s salts (Life Technologies, Gaithersburg, MD) supplemented with 5% FBS (HyClone Laboratories, Logan, UT) and gentamicin (50 μg/ml; Life Technologies). These cells are morphologically consistent with a fibroblast phenotype, and express the fibroblast markers collagen and vimentin. They do not express CD45, factor VIII, or cytokeratin. Fibroblast strains were passaged with a motor (Kontes, Vineland, NJ) at 15 cm2 flasks (Costar, Cambridge, MA). Adherent monolayers of fibroblasts were dissociated with a 1:1 solution of sodium EDTA (0.1% (w/v); Sigma-Aldrich, St. Louis, MO) and trypsin (0.05% (w/v); Life Technologies). All fibroblasts were maintained in a humidified 7% CO2 incubator. In all experiments, cells were used between passage number 4 and 15. Three normal and four fibrotic strains of human lung fibroblasts were used in these experiments.

Reverse transcriptase-PCR

Normal lung fibroblasts (NFL) and fibrotic lung fibroblasts (FLF) were grown in 100-mm tissue culture plates (Costar) as previously described. Total cellular RNA was extracted with TRI- Reagent (1 ml/106 cells; Molecular Research Center, Cincinnati, OH) as per manufacturer’s protocol. Lung tissue from normal and fibrotic areas was dissected into small pieces and homogenized with a pellet pestle motor (Kontes, Vineland, NJ) in TRI-Reagent before proceeding with the manufacturer’s protocol for RNA isolation. RNA from each sample was resuspended in diethylpyrocarbonate-treated water and solubilized by heating to 55°C for 10 min. The concentration of total RNA was quantified by spectrophotometry and treated with DNase (2 U/reaction; Life Technologies) to eliminate any contaminating genomic DNA. Three micrograms of RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (200 U/reaction, Life Technologies) and an oligo(dT) primer (Pharmacia, Piscataway, NJ). A set of replicate samples was generated in which reverse transcriptase was withheld from the reaction; these served as negative controls. The PCR consisted of cDNA generated as previously described (10 μl/reaction), PCR buffer (Boehringer Mannheim, Indianapolis, IN), deoxyribonucleotide-specific primers (1 μM each), and TaqDNA polymerase (2.5 U; Boehringer Mannheim) in a total volume of 50 μl. Samples underwent 40 cycles of amplification in a PTC-200 DNA Engine thermal cycler (MJ Research, Watertown, MA). The protocol included denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and primer extension at 72°C for 1 min. The primers used were either polymerase chain reaction (PCR) sequences were 3′-TTC GAG TAA GCC AAA GGA CGT and 5′-TAG DNA polymerase (2.5 U; Boehringer Mannheim) in a total volume of 50 μl. Samples underwent 40 cycles of amplification in a PTC-200 DNA Engine thermal cycler (MJ Research, Watertown, MA). The protocol included denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and primer extension at 72°C for 1 min. The primers used were either polymerase chain reaction (PCR)

Immunoprecipitation and Western blotting

Confluent monolayers of fibroblasts were lysed in modified RIPA buffer (Tris-HCl 50 mM, pH 7.4, 1% gelatin, 1% sodium deoxycholate 0.25%, EDTA 1 mM, and protease inhibitor mixture; Sigma-Aldrich) allowed to freeze/thaw one time and then centrifuged (14,000 rpm, 10 min) to remove the insoluble material. In some experiments fibroblasts were either left untreated or were stimulated with human IFN-γ (500 U/ml; Genzyme, Cambridge, MA) or human IL-13 (50 ng/ml; R&D Systems, Minneapolis, MN) for 48 h before lysis. Each lysate was precleared using protein G-Sepharose (Amersham Biosciences, Piscataway, NJ) for 1 h at 4°C. Total protein concentration of the precleared lysate was quantified by the bicinchoninic acid method (Pierce, Rockford, IL). Human lung tissue was processed in a similar manner with the addition of a homogenization step after lysis in RIPA buffer. Equivalent amounts of total protein from each precleared sample were immunoprecipitated (2 h) using a monoclonal (Calbiochem, La Jolla, CA) or a polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA) anti-human CD154 Ab, cross-linked to protein G-Sepharose beads (Seize-X Immunoprecipitation kit; Amersham Biosciences). After extensive washing the immunoprecipitates were eluted from the beads by the reduction of pH. As a control, insect cell membranes expressing human
CD154 were immunoprecipitated with or without the addition of the blocking peptide (Santa Cruz Biotechnology). These membranes were prepared from human CD154 recombinant baculovirus-infected (obtained from Dr. M. Kehry, Boehringer Ingelheim, Ridgefield, CT) S21 insect cells. The products were electrophoresed on 12% SDS-PAGE, transferred to nitrocellulose (Hybond C extra; Amersham Biosciences), and probed with mouse anti-human CD154 (MK13A4; a generous gift from Dr. M. Kehry) followed by an HRP-conjugated goat anti-mouse secondary Ab (Amersham Biosciences). Visualization of bands was achieved using the ECL kit (Amersham Biosciences) and exposing the blots to x-ray film (Laboratory Product Sales, Rochester, NY). Two sets of m.w. standards were used to help determine the size of the bands obtained: a prestained low m.w. standard (Bio-Rad, Hercules, CA) and Cruz Markers (Santa Cruz Biotechnology). The density of the bands was determined using Kodak Digital Sciences software.

**Immunocytochemistry**

Fibroblasts were seeded in 8-well chamber slides (Lab-Tek; Nalge Nunc International, Naperville, IL) at a concentration of 1–5 × 10^6 cells per well in culture medium supplemented with 5% FBS. Cells were allowed to adhere overnight after which duplicate wells either received MEM (5% FBS) or MEM (5% FBS) plus human IL-13 (50 ng/ml) or human IFN-γ (500 U/ml), as illustrated below. Following stimulation, cells were washed with PBS, then PBS with 0.1% Tween 20, and treated with 3% H_2O_2 in methanol, blocked in 5% horse serum and incubated overnight at 4°C with either mouse anti-human CD154 (1 μg/ml; Calbiochem) or isotype-specific control Ab mouse IgG1 (1 μg/ml; CalTag Laboratories, Burlingame, CA). Biotinylated anti-mouse Ab (1:200; Vector Laboratories, Burlingame, CA) was used as a secondary Ab and streptavidin-HRP (1:500; Bio-Rad) was added as the substrate. Staining was visualized with a 3-aminophenyl-9-ethylcarbazole staining detection kit (Zymed Laboratories, San Francisco, CA) and photographed using an Olympus BX40 microscope and Sony digital CCM camera.

**Flow cytometry analysis**

Fibroblasts were grown in 100-mm tissue culture plates and prepared for flow cytometry by washing the cell suspension in PBS with 0.1% sodium azide and 1% BSA. For cell surface CD154 staining, cells were incubated directly with the FITC-conjugated mouse anti-human CD154 (Calbiochem). Incubation of cells with FITC-conjugated mouse IgG1 (BD Pharmingen, San Diego, CA) served as a negative control. Intracellular staining was accomplished by first fixing the cells in 2% paraformaldehyde followed by permeabilization with 0.5% saponin in PBS with 0.1% sodium azide and 1.0% BSA and then the addition of either the FITC-conjugated anti-human CD154 or the isotype control Ab. After thorough washing, cells were resuspended in PBS with 0.1% sodium azide and 1.0% BSA and analyzed on a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Data analysis was performed using Becton Dickinson CellQuest software and was based on at least 10,000 events for each specimen.

**Laser confocal microscopy**

Fibroblasts were grown on glass chamber slides (Falcon, Franklin, NJ) under normal culture conditions. Chamber slides were washed with PBS and cells were fixed with 2% paraformaldehyde. Fibroblasts were permeabilized for intracellular staining with 0.5% saponin in PBS with 0.1% sodium azide and 1.0% BSA. The permeabilized cells were then incubated with either a PE-conjugated anti-human CD154 (BD Pharmingen) or the isotype control Ab (BD Pharmingen). After thorough washing, the slides were photographed under the ×40 objective of a Zeiss laser confocal microscope.

**Immunohistochemistry**

Lung tissues evaluated included those from patients with IPF-UlP, sarcoidosis, or were from tissues from histologically normal lung. Formalin fixed, paraffin embedded, 5-μm sections of human lung tissues were prepared and staining initiated by deparaffinizing sections in xylene followed by rehydration in graded alcohols and blocking endogenous peroxidase activity with H_2O_2. Sections were incubated in 5% horse serum to block nonspecific binding sites and then incubated with either anti-human CD154 (1 μg/ml; Calbiochem) or an isotype-matched control Ab (1 μg/ml; CalTag Laboratories) at 4°C overnight. Biotinylated anti-mouse Ab (1:200; Vector Laboratories) was used as a secondary Ab and streptavidin-HRP (1:500; Bio-Rad) was added as the substrate. Staining was visualized with 3-amino-9-ethylcarbazole staining detection kit (Zymed Laboratories) and photographed using an Olympus B ×40 microscope and Sony Digital CCM camera.

**Soluble CD154 ELISA**

Soluble CD154 was measured in both plasma and bronchoalveolar lavage (BAL) fluid using a sandwich ELISA ( Bender MedSystems, Vienna, Austria). All patients gave written informed consent for all procedures, which were approved by the University of Rochester Research Subjects Review Board.

**Plasma collection and preparation**

Ten milliliters of peripheral blood was drawn from the antecubital fossa veins of five healthy control subjects and six patients with a definite diagnosis of the UIP form of IPF. Blood was collected into a heparinized tube and then centrifuged at 2500 rpm for 10 min. Platelet-poor plasma was removed from the cell pellet and stored at −70°C for later analysis of soluble CD154.

**BAL collection and preparation**

Five healthy control subjects and three patients with a definite diagnosis of the UIP form of IPF underwent bronchoscopy, and BAL was collected using standard techniques. In brief, the bronchoscope was introduced through the nose and wedged in the right middle lobe or lingua, and 3 aliquots of 50 ml of 0.9% saline was instilled and immediately gently aspirated. Recovered fluid was pooled. Fluid not required for diagnostic studies was centrifuged and 1400 rpm for 10 min. Fluid was then removed from the resulting cell pellet and stored at −70°C for later analysis of soluble CD154.

**Results**

**CD154 mRNA expression by normal and fibrotic human lung fibroblasts**

Primary strains of fibroblasts originating from normal and IPF fibrotic human lung tissue were examined for the expression of CD154 mRNA. RT-PCR analysis was performed by reverse transcribing equivalent amounts of DNase-treated total cellular RNA and amplifying the resulting cDNA with primers specific for human CD154 and the housekeeping gene β-actin. Samples prepared in the absence of reverse transcriptase failed to generate any product (data not shown). A representative ethidium bromide-stained agarose gel depicting the CD154 amplification products is shown in Fig. 1A. The corresponding β-actin controls are shown in Fig. 1B. Both normal and fibrotic lung fibroblasts express CD154 mRNA.

**Intracellular localization of CD154 in human lung fibroblasts**

In the next series of analyses, we began to explore whether or not CD154 protein was expressed by human lung fibroblasts. NLF were stained with a mAb specific to CD154 or with an isotype control Ab before analysis by flow cytometry. To our surprise, CD154 was not detected on the surface of lung fibroblasts. However, when cells were stained for intracellular expression of CD154, it was easily detected by flow cytometry. Histograms (log fluorescence intensity vs cell number) showing surface (0% positive) and intracellular (22% positive) staining patterns for a NLF strain are shown in Fig. 1C.

**FIGURE 1.** Fibroblasts from normal and fibrotic human lung express CD154 mRNA. Total cellular RNA was isolated from primary strains of lung fibroblasts and equivalent amounts were reverse transcribed into cDNA. The cDNA was amplified using specific primers for human CD154 and human β-actin as described in *Materials and Methods*. PCR products representing CD154 (787 bp) (A) and β-actin (539 bp) (B) were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining. Two strains of NLF and two strains of IPF FLF are shown. +CNTL, CD154 transfected epithelial cell line (positive control).
strain are presented in Fig. 2A. To further assess the intracellular staining pattern for CD154, laser confocal microscopy was used. The photomicrographs shown in Fig. 2B depict these results. No staining was detected when an isotype control Ab was used, (Fig. 2, Bb). When a mAb directed against CD154 was used, staining (red) was visible in some but not all of the cells (Fig. 2, Bd). Fig. 2, Ba and Bc represent the phase contrast images of the fields represented in Fig. 2, Bb and Bd, respectively. The overlay of Fig. 2, Bc plus Bd is presented. The staining pattern observed revealed that CD154 is mainly confined to the perinuclear region of the cytoplasm, however, surface CD154 was detected on tiny surface projections of fibroblasts that help attach them to the plates (Fig. 2, green arrowhead in the inset). Because surface CD154 expression was not detected by flow cytometry it was concluded that trypsinizing fibroblasts for flow cytometry likely clips off the small CD154-positive cell membrane pieces. It also became clear from these data that there is heterogeneity of expression of CD154 within a fibroblast strain. Fig. 2, Bc and Bd show CD154-positive (white arrowhead), as well as negative cells (black arrowhead).

**FIGURE 2.** CD154 is localized intracellularly in human NLF. A, Human lung fibroblasts were stained for surface (left panel) or intracellular (right panel) expression of CD154 and analyzed by flow cytometry. Specific staining (open histogram) was compared with staining with an isotype control Ab (shaded histogram). CD154 was not detected on the surface of lung fibroblasts. Intracellular expression of CD154 was found in 22% of the cells. B, Intracellular localization of CD154 was confirmed by laser confocal microscopy and photographed using the ×40 objective. Phase contrast (a) and fluorescence (b) represent isotype-control staining. The cells in phase contrast (c) and fluorescence (d) were stained with an anti-CD154 Ab. CD154 staining (red) is evident in some, but not all of the cells. This is made more obvious by the overlay of the phase contrast and the fluorescence images (c and d). White arrowheads point to CD154-positive cells, black arrowheads point to CD154-negative cells. CD154 staining was observed to be mainly confined to the cytoplasm although some tiny cellular projections also stained (green arrowhead). An inset (c+d) shows an enlargement of one such area.
CD154 expression by NLF and FLF

It has previously been shown that fibroblasts from the human lung can be induced to increase expression of CD40 under inflammatory conditions (i.e., treatment with IFN-γ) (17, 18). The data reported in this study indicate that lung fibroblasts also express CD154. The CD40-CD154 pathway is known to play an important role in the activation of a number of cell types including fibroblasts and has been postulated to play a role in the development of fibrosis (17, 31, 36, 37). We next asked whether or not there was altered expression of CD154 in FLF vs NLF.

Several approaches were used to determine the levels of CD154 in NLF vs FLF. We first identified the 31 kDa and 33 kDa CD154 specific bands using the CD154-positive control (Fig. 3A) immunoprecipitated with a mAb (Fig. 3A, lane 1) with a polyclonal Ab (Fig. 3A, lane 2), or with the polyclonal Ab preabsorbed with blocking peptide (Fig. 3A, lane 3). In Fig. 3B, the CD154 immunoprecipitation products of two NLF and two FLF strains are presented. In all lung fibroblasts evaluated, both 33 kDa and 31 kDa bands corresponding to CD154 were detected. There was variation in the level of expression of CD154 protein among the different fibroblast strains, even though equal amounts of protein lysate was used. Interestingly, we observed that fibroblasts originating from fibrotic areas of the human IPF lung (FLF) had a greater amount of CD154 than their normal counterparts (NLF). These data are also presented as the relative intensities of the bands as determined by densitometric analysis (Fig. 3C). In another experiment the immunoprecipitated protein from one of the fibroblast strains was transferred to polyvinylidene difluoride membrane and stained with Coomassie blue. The 33 kDa protein band was excised and subjected to N-terminal sequencing at the University of Rochester Microsequencing Facility. The sequence identified corresponds to the known sequence of human CD154 (data not shown).

Differences were also detected in expression of CD154 using several staining techniques. Flow cytometric analysis was used to evaluate the levels of intracellular CD154. Representative histograms showing log fluorescence intensity vs cell number are shown in Fig. 4A. A NLF strain (Fig. 4A, left) was observed to have 32% of the cells expressing CD154 whereas a fibrotic strain (Fig. 4A, right) had 72% CD154+ cells. Immunocytochemical staining for CD154 (Fig. 4B) also confirmed the Western blot data. Fibroblasts were grown on chamber slides and stained for intracellular CD154 using a mAb as described in Materials and Methods. A greater number of positive cells were visualized and a greater intensity of intracellular CD154 staining (red staining) in FLF as compared with NLF.

Differential regulation of CD154 expression by IFN-γ and IL-13

It was of great interest to discover that human fibroblasts express CD154 and that those originating from fibrotic regions of the lung contain more CD154. We next addressed whether CD154 expression by fibroblasts can be regulated by inflammatory mediators. We tested several cytokines including TNF-α, IL-1β, IL-4, IL-13, and IFN-γ, all of which have been shown to activate fibroblasts (17–19, 43). We observed no differences in CD154 expression when lung fibroblasts were treated with TNF-α, IL-1β, or IL-4 (data not shown). However, lung fibroblasts stimulated with IFN-γ showed a marked decrease in CD154 protein expression as detected by Western blot (Fig. 5), by immunohistochemistry (data not shown), and by FACS analysis (data not shown). Fig. 5 shows representative Western blots for CD154 immunoprecipitated from

**FIGURE 3.** Human lung fibroblasts express the 33 and 31 kDa forms CD154. A. Immunoprecipitates of CD154 from insect cell membranes expressing human CD154 with a monoclonal anti-human CD154 (lane 1), a polyclonal anti-human CD154 (lane 2), and a polyclonal anti-human CD154 (lane 3) preabsorbed to the specific blocking peptide. Arrows point to the CD154 specific bands identified as 33 and 31 kDa. B. Equal amounts of total protein were immunoprecipitated from two NLF and two FLF strains using the monoclonal anti-human CD154. All strains of lung fibroblasts were identified to have both the 33 and 31 kDa bands specific for CD154. C. Densitometric analysis of the CD154 specific bands confirms that both fibrotic strains of lung fibroblasts have an enhanced level of 33 and 31 kDa forms of CD154 as compared with their normal counterparts. These experiments were repeated three times with consistent results.
a NLF (Fig. 5A) and a FLF (Fig. 5B) strain. Densitometric analysis of each band is presented as the percentage of relative intensity as compared with unstimulated (no IFN-γ) controls for that band. Both strains were cultured under normal conditions in either medium alone or medium with 500 U/ml IFN-γ. After 24 h, cell lysates were prepared and an equal amount of total protein was immunoprecipitated, as previously described. In both strains, the unstimulated cells expressed both the 33 and 31 kDa forms of CD154 protein. More CD154 is detected in the fibrotic strain, consistent with the data presented in Fig. 3. In addition, it was observed that cells that were treated with IFN-γ had a markedly reduced level of CD154 (both the 33 and 31 kDa) compared with their unstimulated counterparts. This was true for the normal and the fibrotic strains of lung fibroblasts tested.

Lung fibroblasts that were cultured with the type 2 cytokine IL-13 also exhibited changes in CD154 expression. This effect was opposite to that of IFN-γ, in that IL-13 increased the CD154 levels in human lung fibroblast. Lung fibroblasts were cultured with either medium alone or medium supplemented with 50 ng/ml IL-13. At 48 h, cells were harvested and stained for intracellular CD154. Fig. 6 shows histograms of log fluorescence intensity of CD154 staining vs cell number representing one NLF (Fig. 6, left panel) strain and one FLF (Fig. 6, right panel) strain. In both cases the IL-13 stimulated cells (Fig. 6, dashed line) showed a peak shift compared with unstimulated cells (Fig. 6, solid line) and a corresponding increase in the mean fluorescence intensity of the CD154 staining. The mean fluorescence intensity changed from 12 to 19 for NLF strain and from 28 to 58 for the FLF strain. An increase in the percentage of positive cells after IL-13 stimulation was observed but was more pronounced in the NLF (25–35%) vs the FLF (72–75%) strain. Similar results were obtained using immunocytochemical analysis (data not shown). Although, IL-4 and IL-13 have been shown to act through at least some of the same receptors and often have similar effects (43, 44), we did not observe any change in CD154 expression after IL-4 stimulation.

Expression of CD154 in human lung tissue

Our in vitro findings led us to explore the expression of CD154 in the human lung. We initiated these studies by obtaining samples of lung tissues from surgical resections. These tissues were from either normal or fibrotic regions of lung and were subjected to mRNA and Western blot analysis. Total RNA was isolated from
the lung tissues via TRI-Reagent, as described in Materials and Methods, and an equal amount of DNase-treated RNA from each tissue was subject to RT-PCR with human CD154-specific primers or β-actin-specific primers as a control. A representative gel of the resulting products is shown in Fig. 7A. Two fibrotic tissues (Fig. 7A, lanes 1 and 2) and two normal tissues (Fig. 7A, lanes 3 and 4) are presented. Interestingly, CD154 mRNA was detected in all lung tissues examined. However, fibrotic lung tissues (Fig. 7A, lanes 1 and 2) consistently had a greater amount of CD154 product amplified than their normal counterparts (Fig. 7A, lanes 3 and 4). β-actin is evenly detected across all of the lanes. Although these results are semiquantitative, they support that CD154 interaction is a potent activator of fibrosis in the dysregulated process that results in fibrotic lung tissue.

In view of our findings that primary strains of human lung fibroblasts express CD154, we next investigated whether fibroblasts from human tissue could be stained in situ for CD154. To address this, paraffin embedded sections of human lung from normal histological specimens or from UIP-IPF or fibrotic sarcoidosis were evaluated. IPF tissue contained abundant fibroblasts in the scarred areas. Sarcoid lung was also studied as granulomas consist of reactive centers containing macrophages, giant cells, and T lymphocytes surrounded by palisading fibroblasts and dense collagen.

Immunohistochemical analysis of CD154 expression was performed on three normal, three IPF, and three sarcoid lung sections. Fig. 8 details these results. Fig. 8, A, D, and G show the staining pattern for CD154 (red), with arrows pointing to some of the CD154-positive fibroblasts. Fig. 8 (middle) represents the background staining observed with an isotype-control matched mAb. Fig. 8, C, F, and I are trichrome staining of these tissues. Trichrome stains collagen blue and allows visualization of the fibroblast-rich areas and the lung histology. In contrast to the orderly architecture of the normal lung (Fig. 8C) that of IPF is disarranged exhibiting densely packed fibroblasts and dense collagen (Fig. 8F). The fibrosis around the sarcoid granuloma is clearly visible by the thick layer of collagen and its embedded fibroblasts (Fig. 8I). The normal lung (Fig. 8A) shows a low level of staining, with CD154 being expressed in at least some of the fibroblast areas (Fig. 8, arrows) and possibly within some alveolar macrophages. Clearly, there is more staining with the anti-CD154 Ab in the fibrotic regions of both the IPF (Fig. 8D) and sarcoid (Fig. 8G) lung sections as compared with normal (Fig. 8A). Arrows point out some of the CD154 staining fibroblasts within the collagen of these sections. Most clearly visible are those in sarcoid areas surrounding the sarcoid granuloma (Fig. 8G) in which individual fibroblasts can be discerned and clearly seen to express CD154. CD154 staining was also observed in the granulomas. This likely represents CD154 within macrophage-like cells. No staining was observed with an isotype-matched control Ab as shown in Fig. 8, B, E, and H.

CD154 has been reported to also exist in a soluble/shed form. Using an ELISA we measured the amounts of soluble CD154 present in both platelet-poor plasma and BAL fluid from patients with IPF, as well as from normal control subjects. Fig. 9 clearly shows that plasma (Fig. 9, left) and BAL (Fig. 9, right) from patients with IPF has significantly higher levels of CD154 than normal controls.

Discussion

CD154 was first described as a protein expressed exclusively by activated T lymphocytes and as the natural ligand for CD40. CD40-CD154 interactions were found to be essential for generating effective humoral immunity, but this was believed to be the extent of function of this receptor-ligand pair. The role of this

FIGURE 6. IL-13 stimulation enhances CD154 expression in human lung fibroblasts. Human NLF (A) and FLF (B) were cultured in either medium alone or with human IL-13 (50 ng/ml). After 48 h, fibroblasts were stained for intracellular CD154 and analyzed by flow cytometry. Both NLF and FLF responded to IL-13 stimulation showing an increased mean fluorescence intensity of CD154 staining as compared with unstimulated cells. This experiment was repeated three times with similar results.
pathway has to be reexamined in light of recent findings that both CD40 and CD154 expression are detected on other cell types. The CD40-CD154 pathway has been implicated in the development of lung injury and fibrosis. In this study, we examined the expression of CD154 by human lung fibroblasts and found that primary strains of fibroblasts derived from normal and fibrotic tissues express CD154. Moreover, CD154 expression was found to be elevated in fibrotic fibroblasts and fibrotic lungs in situ as compared with non-fibrotic controls. In addition, soluble CD154 levels were significantly increased in the plasma and BAL of IPF patients as compared with normal controls. We hypothesize that lung fibroblasts, initially activated by infiltrating T lymphocytes, are involved in the perpetuation of the inflammatory and wound healing responses by autocrine and paracrine activation via the CD40-CD154 pathway.

RT-PCR analysis of total cellular RNA revealed detectable basal levels of CD154 mRNA in both NLF and FLF (Fig. 1). By using immunofluorescent staining with flow cytometry (Fig. 2A) and confocal microscopy (Fig. 2B) techniques, we demonstrated that CD154 protein is also expressed in human lung fibroblasts. Interestingly, we found that the majority of CD154 is localized to the cytoplasm rather than the cell membrane. This is consistent with observations by others that a subset of lymphocytes and platelets have large pools of preformed CD154 inside of them (6–8, 45).

There is now ample evidence indicating that fibroblasts originating from a diseased tissue can display properties different from those originating from normal tissue of the same anatomical origin (46, 47). Our data further reinforces this concept by showing that FLF express higher levels of CD154 compared with NLF (Fig. 3). This finding was confirmed by immunoprecipitation and Western blotting (Fig. 3B), and flow cytometry (Fig. 4A) and immunocytochemistry (Fig. 4B). We were able to confirm the relevance of

FIGURE 8. Human lung fibroblasts express CD154 in situ. Lung tissue samples from normal (A–C) or fibrosis due to either IPF (D–F) or sarcoidosis (G–I) were stained for CD154. Serial tissue sections were stained with anti-CD154 Ab (A, D, and G) or an isotype control Ab (B, E, and H). CD154 staining is visualized by the reddish/brown color. Arrows show some of the fibroblasts that are CD154-positive. Trichrome staining (C, F, and I) is used to visualize collagen (blue) and the fibroblast-rich areas.

FIGURE 9. Plasma and BAL fluid from patients with IPF have elevated levels of soluble CD154 compared with controls. Plasma and BAL were collected from healthy control subjects and from patients with IPF, and the levels of soluble CD154 assayed by ELISA as described in Materials and Methods. The mean plasma level (left panel) of soluble CD154 in patients with IPF is elevated compared with the mean of healthy control subjects (p = 0.006; t test). The mean BAL fluid level (right panel) of soluble CD154 is elevated in patients with IPF compared with healthy controls (p = 0.05; Mann-Whitney U test)
our in vitro cultured fibroblasts data by investigating CD154 expression associated with normal and fibrotic lung tissues. These results mimicked those of the primary fibroblast cultures. Higher levels of CD154 mRNA and protein were detected by RT-PCR and Western blot, respectively, in fibrotic lung tissues as compared with normal lung (Fig. 7). These data are interesting but do not confirm that the CD154 expression observed is from the resident fibroblasts. To answer this we used immunohistochemical analysis of CD154 expression in lung tissues in situ. Interestingly, fibroblasts within the lung architecture detectable levels of CD154. Normal lung tissue sections displayed the characteristic lace-like architecture (Fig. 8C) and had a low level of CD154 staining associated with them (Fig. 8A). In contrast, the fibrotic lungs had a vastly different appearance. There was an abundance of collagen throughout the tissues, and many more fibroblasts visible within the ECM (Fig. 8, F and I). The fibroblasts within these lungs were also more abundantly stained for CD154 (Fig. 8, D and G).

Human lung fibroblasts are heterogeneous with respect to expression of CD154. We observed that in primary cultures of lung fibroblasts there were cells that stained positive for CD154 and those that were negative for this protein (Figs. 2B and 4). Heterogeneity among fibroblasts has been previously described based on the expression of the surface marker Thy-1 (48–50). In contrast, lung fibroblasts homogeneously express CD40 (17, 18), suggesting that nearly all fibroblasts are capable of receiving signals via CD40. Our findings suggest that a subset of fibroblasts that expresses CD154 exists in the lung, and that this subset is capable of transducing signal to neighboring CD40-positive cells. Basal levels of CD40 are low in human NLF, but this can be up-regulated with inflammatory mediators such as TNF-α, IL-1β, and IFN-γ (18). IFN-γ has shown to have suppressive effects on the proliferation and collagen production of fibroblasts (51, 52). In contrast to IFN-γ effect on CD40 expression, we have found that this cytokine dramatically decreases the amount of CD154 expressed by lung fibroblasts (Fig. 5). This finding may help explain the effectiveness of IFN-γ treatment observed in a preliminary study of IFP (53). In this study, the authors reported a significant improvement in lung function and gas exchange in patients treated over the course of a year with prednisolone and IFN-γ as compared with treatment with prednisolone alone. Recent data (Inter- mune, unpublished observations) from a phase 3 trial also suggests a mortality benefit from therapy with IFN-γ in IPF patients.

IL-13, a cytokine that has been described as profibrotic (43, 44, 54), increases the levels of detectable CD154 in human lung fibroblasts (Fig. 6). IL-13 is implicated in the pathogenesis of various diseases characterized by fibrosis. Increased levels of IL-13 have been detected in the BAL of patients with asthma and different forms of pulmonary fibrosis (55, 56). A transgenic mouse model has been used to demonstrate that over-expression of IL-13 in the lung generates pulmonary fibrosis (57). IL-13 receptors have been identified on human fibroblasts. The IL-13R is a heterodimer that shares one common receptor chain and some common signal transduction pathways with the IL-4R (43). This could partly explain why IL-13 and IL-4 share some overlapping biological activities. However, some differences have been observed in IL-4/IL-13 receptor chain expression or usage among human lung and skin fibroblasts, as well as differences in responses to stimulation with these cytokines. For example, although both IL-4 and IL-13 potently up-regulate total collagen production in skin fibroblasts, IL-13, but not IL-4, was shown to induce procollagen 3α1 gene expression (54). These results are relevant to our observation that IL-13, but not IL-4, increased CD154 expression in human lung fibroblasts. The concept that IL-13 is a profibrotic cytokine, combined with our results showing that this cytokine can increase CD154 expression by fibroblasts leads us to hypothesize that an IL-13-rich microenvironment could promote CD154 over-expression in lung fibroblast subpopulations allowing them to act as effector cells in the pathogenesis of chronic inflammation and lung remodeling.

Another exciting observation from this study was that patients with IPF had increased levels of circulating soluble CD154 in their plasma and BAL fluid compared with control subjects (Fig. 9). A link between soluble CD154 levels and disease has already been established. For example, patients with unstable angina have higher concentrations of soluble CD154 than those with stable angina or healthy volunteers (58). Soluble CD154 has been reported to be secreted and/or shed from activated lymphocytes and platelets. Platelets are a major source of CD154 in circulation. Soluble CD154 has been shown to be generated from platelets by the proteolytic cleavage of CD154 from the cell membrane after binding to platelet-expressed CD40 (59). Lung fibroblasts may use a similar mechanism for shedding CD154 into the microenvironment. The receptor for CD154, CD40, is up-regulated on fibroblasts during inflammation and is constitutively expressed on platelets (58, 60). Thus, fibroblast CD40 in combination with platelets, may act to perpetuate the inflammatory state that is conducive to scarring and fibrosis. Monitoring soluble CD154 levels of patients at risk for developing fibrosis may prove to be a useful biomarker and diagnostic tool.

Our results clearly demonstrate for the first time that human lung fibroblasts express CD154 in vitro and in situ. We conclude from our data that the level of CD154 expression by lung fibroblasts is increased in fibrotic compared with normal tissue. In light of our novel finding that fibroblasts express CD154 we postulate a model of autocrine and paracrine activation of lung fibroblasts via the CD40-CD154 pathway. Lung fibroblasts may initially become activated by infiltrating cells during the inflammatory phase of the wound healing process. However, when the inflammatory cells are gone, or no longer express CD154, a subset of lung fibroblasts can predominate and activate neighboring resident cells through CD40. If this mode of activation goes unchecked it may result in the chronic activation of fibroblasts and tissue remodeling that precedes the development of pulmonary fibrosis. Our data also support the hypothesis that fibroblasts are not merely resident structural cells but also act as dynamic effector cells that are capable of participating in inflammatory and remodeling processes.

References


