STAT3 is a latent transcription factor that plays a role in regulating fibroblast function in fibrotic lung diseases. To further understand the role of STAT3 in the phenotypic divergence and function of human lung fibroblasts (LFs), we investigated the effect of basal and cytokine-induced STAT3 activity on indices of LF differentiation and activation, including expression of α-smooth muscle actin (α-SMA), collagen, and adhesion molecules Thy-1/CD90 and α, β1, and β3 integrins. We identified a population of fibroblasts from usual interstitial pneumonia (UIP)/idiopathic pulmonary fibrosis (IPF) lungs characterized by constitutively phosphorylated STAT3, lower proliferation rates, and diminished expression of α-SMA, Thy-1/CD90, and β3 integrins compared with control LFs. Staining of UIP lung biopsy specimens demonstrated that phosphorylated STAT3 was not present in α-SMA–positive fibroblastic foci but was observed in the nuclei of cells located in the areas of dense fibrosis. STAT3 activation in LFs did not significantly influence basal or transforming growth factor β1–induced collagen I expression but inhibited expression of α-SMA, Thy-1/CD90, and αβ3 integrins. Suppression of STAT3 signaling diminished resistance of IPF LFs to staurosporine-induced apoptosis and responsiveness to transforming growth factor β, but increased basal α-SMA and restored β3 integrin expression in LFs via an ALK-5–dependent, SMAD3/7-independent mechanism. These data suggest that STAT3 activation regulates several pathways in human LFs associated with normal wound healing, whereas aberrant STAT3 signaling plays a critical role in UIP/IPF pathogenesis. (Am J Pathol 2012, 180:1398–1412; DOI: 10.1016/j.ajpath.2011.12.022)
phologic characteristics, proliferative potential, response to inflammatory mediators and extracellular matrix (ECM), biosynthetic capacity, and reduced cell surface expression of Thy-1/CD90 and caveolin-1. However, the molecular mechanisms and transcription factors regulating activation, differentiation, persistence, and clearance of human lung myofibroblasts, either in normal wound repair process or in IPF, remain largely unknown.

Current data suggest that myofibroblasts are the primary cell type responsible for the excessive synthesis and deposition of collagen and other ECM proteins and the resultant tissue remodeling in a multitude of fibrotic diseases of the lung, including UIP/IPF. α-SMA expression is the most well-known characteristic and widely used marker of myofibroblasts. Although several molecules have been shown to induce α-SMA and promote the appearance of myofibroblasts in vitro and in vivo, the best studied pathway is SMAD-dependent signaling induced by transforming growth factor (TGF)-β. However, recent findings suggest that knocking out α-SMA expression surprisingly worsens experimental fibrosis in mouse kidney and lung. Moreover, we and others have recently reported that a normal wound healing process of human lung parenchyma is associated with activation of TGF-β signaling and persistence of myofibroblasts without an accompanying fibrogenic response. Thus, these observations strongly support the idea that disruption of the intrinsic program of myofibroblast differentiation and activation in injured lungs induces aberrant wound healing, which ultimately leads to fibrosis.

STAT3 is a ubiquitously expressed latent cytoplasmic protein that is transiently activated by a large number of different ligands, including the IL-6 family cytokines, several growth factors, and nonreceptor (Src-family) tyrosine kinases. Activation of STAT3 has been shown to play critical roles in numerous biological activities, including cell proliferation, migration, survival, and oncogenesis in a tissue- and cell-specific manner. In this context, skin fibroblasts derived from keloids display constitutive activation of STAT3 signaling, and its inhibition reversed the enhanced collagen production and proliferative activity of the cells. In our previous studies, we demonstrated that a specific population of IPF-derived fibroblasts displayed the opposite response to IL-6 stimulation, diminished apoptosis and increased proliferative activity compared with control fibroblasts, and this response was associated with a relative inability to activate STAT3. These conflicting results further support the idea that multiple fibroblast phenotypes might be associated with fibrotic disease initiation and progression. However, the precise role of STAT3 in regulating mesenchymal cell phenotypes during the evolutionary phases of the lung fibrosis is unknown.

To address this, we used several approaches to examine the effects of STAT3 activation on key elements of fibroblast-myofibroblast differentiation and fibrogenesis. We identified a unique subpopulation of lung fibroblasts (LFs) from patients with IPF characterized by constitutively active STAT3 expression, which display a differential gene expression profile. In tissue sections of IPF lungs, we found cells with high levels of phosphorylated STAT3 (pSTAT3) in the areas of dense fibrosis but not in fibroblastic foci. We then generated several in vitro models using primary normal LFs and demonstrated that sustained activation of STAT3 markedly decreased the expression of several key surface receptors and α-SMA, recapitulating the pathological phenotype of the IPF-derived cells. In contrast, inhibition of high basal STAT3 signaling by a dominant-negative STAT3 mutant (STAT3DN) or the STAT3 inhibitor STA-21 enhanced expression of β3 integrin and α-SMA in normal LFs and decreased resistance of IPF LFs to staurosporine-induced apoptosis and TGF-β responsiveness. These findings suggest that STAT3 is a key transcription factor regulating human LF-myofibroblast differentiation and homeostasis and as such likely plays a role in the pathogenesis of UIP/IPF.

Materials and Methods

Human Lung Tissue

This study was approved by the ethics committees of all institutions involved. The IPF lung tissues were obtained from diagnostic lung biopsy specimens or explanted lung during lung transplantation. The IPF patients underwent a history, physical examination, high-resolution computed tomography, pulmonary function tests, and diagnostic lung biopsy. In all cases the pathological diagnosis was UIP and the consensus clinical diagnosis was IPF. Normal human lung tissue was obtained from macroscopically tumor-free lung resections of patients with lung cancer and from healthy transplant donor’s lungs not used for transplantation obtained through the International Institute for the Advancement of Medicine (Edison, NJ).

LF Isolation and Culture

Primary cultures of LFs were derived from distal parenchyma of healthy, nonsmoking transplant donors, patients with lung cancer (controls), and patients with IPF as previously described. In the present study, we identified and characterized LFs isolated from 15 patients with UIP/IPF. Because of the abnormal growth characteristics of IPF LFs, it was impossible to synchronize all these fibroblast lines with the control LF cultures and analyze them simultaneously. Some of the IPF LF cultures grew at such a slow growth rate that we were able to evaluate them only in one independent experiment. Normal human diploid fetal lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium with high levels of glucose and l-glutamine (Invitrogen, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% antibiotics/antimycotics (Invitrogen) [complete medium (CM)]. All primary fibroblast cultures from IPF patients and control groups were used at passages 3 to 6 under the same cell culture conditions. Before experiments, HFL-1 cells and primary LFs were placed in 6-well
plates (BD Bioscience, Mississauga, Ontario, Canada) in CM at a density of 10^6 cells/well and cultured overnight. For STAT3 activation, the recombinant human cytokines IL-6, IL-11, leukemia inhibitory factor, oncostatin M (OSM) (R&D Systems, Minneapolis, MN), and IL-10 (PeproTech, Rocky Hill, NJ) were used. Recombinant, human, biologically active TGF-β, (PeproTech) was used for induction of α-SMA and collagen I expression. Cell-permeable and highly selective STAT3 inhibitory peptide (PpYLTKT-mts, S3IP) was purchased from EMD Chemicals (Gibbstown, NJ) and added to primary LF cultures for 16 to 24 hours. The new STAT3 inhibitor STA-21 was obtained from Enzo Life Sciences International (Plymouth Meeting, PA). HFL-1 cells and primary LFs were exposed to STA-21 for 24 to 72 hours at a concentration of 20 μmol/L. Apoptosis was induced by exposure of primary LFs to staurosporine (0.5 μmol/L) (Sigma-Aldrich, Oakville, Ontario, Canada) for 16 hours in serum-free medium.

Antibodies

Antibodies against STAT3, Smad2/3, and β3 integrin were purchased from BD Biosciences, pSTAT3 (Tyr705) and cleaved caspase-3 antibodies were purchased from Cell Signaling Technology (Danvers, MA). β-Tubulin, α, β3, and Thy-1/CD90 antibodies were purchased from Millipore (Billerica, MA). Human α-SMA and collagen I antibodies were purchased from Sigma-Aldrich and Abcam (Cambridge, MA), respectively. Antibodies against human phospho-Smad3, α, β3, β5 integrin, and pro-caspase-3 were obtained from Epitomics (Burlingame, CA), R&D Systems, Abcam, and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

DNA Constructs, Lentivirus Preparation, and Generation of Stably STAT3-Transduced LFs

The STAT3C-expressing vector was obtained from the nonprofit plasmid repository Addgene (Cambridge, MA) according to a material transfer agreement with Dr. James Darnell (Rockefeller University, New York, NY). The vectors pLent6/V5-GW/STAT3C were constructed using the ViraPower Lentiviral Expression System (Invitrogen) and the pLent6/V5 Directional TOPO Cloning Kit (Invitrogen) according to the manufacturer’s protocols. pLent6/V5-GW/LacZ vector was provided by Invitrogen using a ViraBind Adenovirus Purification Kit (Cell Biolabs, San Diego, CA). Small-volume aliquots of cleared and concentrated viruses were kept at −80°C. In the preliminary experiments with HFL-1 cells and primary LFs, the optimal concentration of viruses for infection was determined (150 to 300 ng of the viral DNA in 1 mL of CM). HFL-1 or LFs were placed in six-well plates and cultured overnight in CM. HFL-1 or LFs were exposed to viruses for 24 hours. After infection, cells were washed with fresh CM and further incubated for additional 24 to 48 hours. Efficiency of the infection was monitored by a fluorescent microscopy detecting GFP-positive cells. Approximately 80% of cultured HFL-1 or LFs were infected after 24 hours of exposure and expressed GFP-STAT3DN or GFP alone as detected by flow cytometry using this infection protocol. Characteristics of the infected LFs are provided in Supplemental Figure S2, A and B (available at http://ajp.amjpathol.org), showing that infection of human LFs with Ad-STAT3WT induced significant activation of STAT3 signaling by enhanced phosphorylation of the overexpressed STAT3 protein. Ad-STAT3DN and Ad-GFP or mock-infected HFL-1 was stimulated with TGF-β1 (5 ng/mL, 48 hours) in the presence of the ALK-5 inhibitor SB505124, and α-SMA mRNA and protein levels were determined by quantitative RT-PCR and immunoblotting, respectively.

Immunoblotting and Protein Expression/Phosphorylation

The expression of STAT3, pSTAT3, Smad 2/3, phospho-Smad3, β-tubulin, α-SMA, collagen I, and β3 and β5 integrins was assessed by immunoblotting as previously described. Briefly, total cell protein extracts were resolved by SDS-PAGE and transferred to nitrocellulose
membranes. Membranes were blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) and probed with the appropriate target antibody. Detection was performed with IR700 and IR800 anti-mouse and anti-rabbit antibodies (Rockland Immunochemicals, Gilbertsville, PA) and the Odyssey Infrared Imaging System (LI-COR Biosciences) using a manufacturer’s protocol. Density of the bands was quantified in two infrared channels independently using Odyssey software 2.1 (LI-COR Biosciences). The results are expressed as a phosphorylated protein/nonphosphorylated protein density ratio or protein/β-tubulin density ratio. An equal amount of protein extract from the HFL-1 cell line stimulated with 10 ng/mL of OSM for 15 minutes was used as an internal standard on each immunoblot for Y705-pSTAT3/STAT3 detection. Densitometry values of this standard were used as a denominator for normalization and percentage calculation of STAT3 activation level in the individual probes.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded non-IPF and IPF lung tissue biopsy samples were obtained from tumor-free specimens of lung resections of patients with lung cancer (n = 4) and from patients with UIP/IPF (n = 3), respectively. Serial sections were blocked for 30 minutes in 10% FBS in Tris-buffered saline (TBS) and incubated with primary rabbit anti-human Y705-pSTAT3 antibody (Cell Signaling Technology) or mouse monoclonal anti–α-SMA antibody (clone 1A4, Sigma-Aldrich) diluted in TBS/0.05% Triton X-100 for 1 hour at room temperature. After sequential washing with TBS, tissue sections were incubated with a biotin-conjugated anti-rabbit secondary antibody (DakoCytomation, Glostrup, Denmark) at 1:200 in TBS/0.05% Triton X-100 for 45 minutes, followed by streptavidin–horseradish peroxidase conjugate (1:200 dilution, DakoCytomation) for 30 minutes at room temperature. pSTAT3 expression was visualized using 3,3’-diaminobenzidine (Sigma-Aldrich), with areas of positive staining shown in brown. Sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted in DePeX mounting medium (Merck, Frenchs Forest, Australia). As a negative control for pSTAT3 expression, primary antibody was omitted. Serial sections of lung tissue biopsy specimens were also stained with the Masson’s trichrome stain for total collagen detection.

**Confocal Laser Scanning Microscopy**

Fibroblasts were cultured on eight-well chamber slides (1 × 10⁴ cells per well), fixed in cold ethanol for 15 minutes, rehydrated, and permeabilized in 0.2% Triton X-100 for 3 minutes. After several washes in PBS, cells were blocked with 5% goat serum at room temperature for 1 hour and then stained with monoclonal mouse antibodies against human αv, β3, and αv, β5 integrins for 2 hours at room temperature. After further washes in PBS, slides were exposed to Alexa Fluor-488–conjugated secondary goat anti-mouse antibody (Invitrogen). Cells were counterstained with DAPI to visualize nuclei. All images were obtained using a Leica AOBS SP2 confocal microscope (Leica Microsystems GmbH, Heidelberg, Germany).

**Real-Time Quantitative RT-PCR**

RNA was obtained from cultured fibroblasts using the RNeasy kit from Qiagen (Valencia, CA) using the manufacturer’s protocols. RNA was then reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. Real-time quantitative PCR reactions were prepared with TaqMan probe–based gene expression assays and TaqMan universal PCR master mix, both purchased from Applied Biosystems (Foster City, CA). Real-time PCR reactions were performed and data analyzed as previously described.⁷

**Flow Cytometry**

Primary LF monolayer was immersed in trypsin/EDTA solution; then cells were collected, washed one time with culture medium (supplemented with 10% FBS), and resuspended in PBS with 10% FBS. LFs after incubation in PBS with 10% FBS for 30 minutes at room temperature were fixed in 2% paraformaldehyde for 10 minutes on ice. Cell surface expression of Thy-1/CD90 was analyzed using an anti-human CD90 monoclonal mouse antibody (clone F15-42-1; Millipore) and normal mouse IgG, (Santa Cruz Biotechnology) as an isotype control, followed by incubation with phosphatidyethanolamine-conjugated goat–anti-mouse F(ab’)₂ (Cedarlane Laboratories, Burlington, ON, Canada). Cell-associated fluorescence was acquired by a Coulter EPICS XL flow cytometer (Beckman Coulter, Mississauga, ON, Canada) and analyzed using WinMDI software version 2.8 (Scripps Research Institute; http://facs.scripps.edu/software.html).

**Statistical Analysis**

Statistical comparisons were performed using analysis of variance followed by Fisher’s protected least significant difference test performed post hoc. For nonparametric analysis, the Mann-Whitney U-test was used. Probability values were considered significant if they were <0.05. All tests were performed using StatView software version 5.0 (SAS Institute Inc., Cary, NC).

**Results**

**IPF LFs Are a Heterogeneous Cell Population with Different Proliferative Activity, Basal STAT3 Signaling, and Gene Expression Profiles**

We have previously demonstrated that STAT3 activation in response to IL-6 was blunted in a small number of IPF LF lines compared with control LFs.¹³ These IPF LF lines were characterized as highly proliferative and resistant to Fas-induced apoptosis.³⁰ In the present study, we identified and characterized LFs isolated from patients with UIP/IPF, which displayed noticeably impaired growth activity and distinct morphologic characteristics compared with control cells.
Doubling time of IPF LF lines was significantly longer compared with control LFs cultured in the same cell culture conditions (Figure 1A). In addition, the IPF LF lines were substantially larger compared with control LFs and displayed stellate morphologic characteristics (Figure 1B). We examined constitutive levels of Y705-pSTAT3 in IPF LF lines and found higher basal STAT3 signaling activity compared with control LFs (Figure 1, C and D). Further analysis revealed that IPF LF lines also expressed significantly less \( \alpha \)-SMA (ACTA2) and \( \beta_3 \) integrin (ITGB3) but more fibronectin-1 (FN1) mRNA at baseline (Figure 1E). There was a trend toward increased basal expression levels of vimentin and collagen 1 \( \alpha_1 \) (COL1A1) in IPF LFs compared with control LFs, but this difference did not reach statistical significance (Figure 1E). Thus, the increased basal pSTAT3 expression level in IPF LFs was associated with a specific phenotype characterized by a markedly decreased expression of \( \alpha \)-SMA and \( \beta_3 \) integrin but increased expression of FN1 and slower growth rate compared with control cells.

**Localization of pSTAT3 Immunoreactive Cells in the Lungs of Patients with UIP/IPF**

We next sought to determine the localization of cells with activated STAT3 signaling in UIP lungs. Figure 2, A–C, shows areas of a dense fibrosis with numerous cells strongly positive for pSTAT3. Regions within fibroblastic foci and surrounding vasculature showed strong staining for \( \alpha \)-SMA, but pSTAT3-labeled cells were not observed (Figure 2, D and E, respectively). Some cells within the alveolar septa of the lungs from non-IPF controls stained positive for pSTAT3 but not as extensively as in IPF lungs (Figure 2, G and E, respectively) and on closer examination appeared to be restricted to alveolar epithelial cells (Figure 2H).

**Modulation of Basal STAT3 Signaling Modifies Collagen I Secretion but Not Gene Expression in IPF LFs**

Thus far, our findings show that IPF LFs express high basal levels of pSTAT3 and FN1 in vitro and are adjacent to areas of dense fibrosis in vivo. We next tested whether activation of STAT3 induced by gp130 agonists is involved in the fibrogenic function of LFs. First, we determined the responsiveness of IPF LFs to gp130-mediated STAT3 signaling. These experiments revealed that both controls LFs and IPF LFs normally respond to IL-10, IL-11, and OSM stimulation; however, several IPF LF cultures displayed diminished responsiveness to IL-6 and...
leukemia inhibitory factor compared with control LFs (Figure 3A). However, stimulation with IL-6 or OSM alone and in the presence of a cell-permeable STAT3 inhibitor peptide did not significantly alter COL1A1 and COL1A2 secretion by control LFs (Figure 3B). Figure 3C shows a trend toward higher basal levels of COL1A1 mRNA in IPF LFs compared with control LFs, although this difference did not reach statistical significance. As expected, TGF-β1 dose dependently increased COL1A1 mRNA in both control LFs and IPF LFs (Figure 3C). In contrast, IL-6 and OSM did not significantly change COL1A1 mRNA expression levels either alone or in combination with TGF-β1 in both IPF LF and control LF cultures (Figure 3C). We also transiently transfected HFL-1 cells with a plasmid-expressing human STAT3 and determined COL1A1 and COL1A2 secretion at baseline and after IL-6 or OSM stimulation. Cells overexpressing STAT3 displayed 90 times higher SOCS3 mRNA expression levels after stimulation, with IL-6 confirming functionality of the STAT3 construct; however, they did not secrete more COL1A1 and COL1A2 either at rest or after cytokine stimulation compared with GFP-transfected controls (see Supplemental Figures S1B and S3 at http://ajp.amjpathol.org). In contrast to normal LFs (Figure 3D), infection of IPF LFs with Ad-STAT3DN had a strong inhibitory effect on basal and TGF-β1–induced COL1A1 and COL1A2 secretion compared with Ad-GFP–infected controls (Figure 3E). The most prominent effect of STAT3 inhibition was seen in 50% of IPF-derived lines predominantly affecting secretion of COL1A2 chain (two of four IPF LFs examined are shown in Figure 3E). Taken together, these findings suggest that STAT3 signaling has little effect on collagen I expression and secretion in normal LFs, but it might be involved in the regulation of collagen I secretion by lung fibroblasts in UIP/IPF.

IL-6 and OSM Down-Regulate TGF-β1–Induced α-SMA Protein in LFs by STAT3-Dependent Mechanism

The observation that IPF LFs showed reduced basal α-SMA gene expression prompted us to ask whether elevated STAT3 signaling was involved. Thus, we stimulated control LFs with IL-6, OSM, or TGF-β1 alone and in combination for 8 hours and measured production of α-SMA by immunoblotting. As expected, TGF-β1 was the most potent inducer of α-SMA expression (12-fold induction over baseline) (Figure 4, A and B). Although IL-6 modestly up-regulated α-SMA production alone, it significantly diminished α-SMA induced by TGF-β1. Exposure to OSM, the most potent activator of STAT3 signaling, had no effect on α-SMA expression alone but had an even more pronounced inhibitory effect on TGF-β1–induced α-SMA expression (Figure 4, A and B). Interestingly, the inhibitory effect of STAT3 activation was not associated with modulation of Smad3 phosphorylation levels either at baseline or induced by TGF-β1 (Figure 4B). To examine this further, we infected LFs derived from normal lung parenchyma with either Ad-STAT3DN or Ad-GFP and measured α-SMA expression after OSM stimulation. We have previously shown that these cells consti-
tutively express enhanced TGF-β1 signaling and α-SMA expression. Figure 4C shows that OSM inhibited basal α-SMA expression in normal LF cultures after 24 hours, whereas blockade of STAT3 signaling by STAT3DN completely abrogated this inhibitory effect, confirming a critical role for STAT3 signaling in OSM-induced inhibition of α-SMA. Moreover, inhibition of basal STAT3 signaling by overexpression of STAT3DN mutant protein significantly
Inhibition of STAT3 Signaling Increases α-SMA Expression in Fibroblasts via an ALK-5–Dependent but SMAD7-Independent Mechanism

The results from the previous experiments suggest that STAT3 has a suppressive effect on basal α-SMA expression of normal LFs that does not appear to involve any inhibitory effects on Smad3. We next investigated the mechanisms underlying this inhibitory effect further. As shown in Figure 5, A and B, inhibition of basal STAT3 signaling by Ad-STAT3DN significantly increased basal and TGF-β1–induced α-SMA gene expression and protein production compared with Ad-GFP–infected or mock-infected cells. ALK-5 inhibition completely abrogated α-SMA expression induced by exogenously added TGF-β1 in Ad-STAT3DN–infected cells, indicating that TGF-β signaling was functional in this model. However, the presence of the ALK-5 inhibitor did not completely abrogate STAT3DN-induced α-SMA expression in the presence or absence of TGF-β1, suggesting TGF-β-independent mechanisms are also involved (Figure 5, A and B). Expression of TGF-β1 mRNA was strongly up-regulated by exogenously added TGF-β1 and was not significantly modified by Ad-STAT3DN, excluding the possibility of STAT3DN increasing autocrine expression of TGF-β1 (Figure 5C). Moreover, we found that in contrast with α-SMA, SMAD7 gene expression level at baseline was not affected after infection with Ad-STAT3DN (Figure 5D). Importantly, Ad-STAT3DN depressed SMAD7 mRNA expression induced by the exogenously added TGF-β1, and this inhibitory effect was completely abrogated by SB505124 (Figure 5D), suggesting a key role of ALK-5 signaling in the SMAD7-mediated feedback loop. We reproduced these findings using the STAT3 small molecule inhibitor STA-21 (see Supplemental Figure S4 at http://ajp.amjpathol.org). Collectively, these results suggest that basal STAT3 signaling is a repressor of α-SMA expression in human normal lung fibroblasts, and blockade of ALK-5 activation, but not SMAD3-mediated signaling, is involved.

STAT3 Activity Reduces α, β3 Integrin Expression

Having established that STAT3 suppresses α-SMA expression, we next determined whether the modifying effects of STAT3 extended to other fibroblast-myofibroblast differentiation markers. In initial experiments, we screened 16 primary LF cultures for basal STAT3 activation levels and found several lines that expressed constitutively a high basal level of pSTAT3 over several repeated passages, similar to that seen in the IPF LF lines. Interestingly, these cells, designated LF-pSTAT3high, were characterized by the absence of α, β3 integrin expression on the cell surface (Figure 6A), as well as low proliferation rates when compared with LF-pSTAT3low (data not shown). In contrast, surface expression of α, β3 integrins in LF-pSTAT3low was slightly higher compared with LF-pSTAT3high (Figure 6A). To determine a causal role for STAT3 in this integrin expression profile, cells were infected with Ad-STAT3DN or STAT3C or treated with the small molecule inhibitor STA-21. As shown in Figure 6B, the higher basal level of α, β3 integrin expression on the cell surface of LF-pSTAT3low was mirrored by...
an enhanced β3-integrin mRNA expression, suggesting a transcriptional effect of STAT3. Furthermore, Ad-STAT3DN significantly up-regulated basal β3-integrin gene expression in both LF-pSTAT3high and LF-pSTAT3low cells. This outcome was confirmed using the selective STAT3 inhibitor STA-21 (see Supplemental Figures S4 and S5 at http://ajp.amjpathol.org). In contrast, neither Ad-STAT3DN (Figure 6C) nor STA-21 treatment (data not shown) significantly altered basal β5-integrin mRNA expression. However, LF-pSTAT3high constitutively expressed higher β3-integrin mRNA levels compared with LF-pSTAT3low (Figure 6C). To further confirm these findings, we forced activation of STAT3 using a STAT3C construct and found decreased expression of β3 integrin in STAT3C-transduced cells when compared with parental noninfected and LacZ-transduced controls (Figure 6D). The increased β3-integrin gene expression of LFs infected with Ad-STAT3DN was paralleled by a significantly elevated β3-integrin protein level determined by immunoblotting (Figure 6E). Thus, these findings suggest that basal STAT3 activity in LFs represses β3 integrin via a transcription-dependent mechanism. In contrast, STAT3 does not appear to be significantly involved in regulating β5-integrin expression.

Expression of Thy-1/CD90 on the Cell Surface of LFs Is Negatively Correlated with STAT3 Activation at Baseline

Thy-1/CD90 expression is associated with distinctive fibroblast phenotypes, and reduction of this cell surface molecule has been found in UIP/IPF-derived LFs.6,12 Furthermore, interaction of αv β3 and αv β5 integrins with Thy-1 is an important mechanism, mediating cell adhesion, communication, and TGF-β signaling.37,38 Therefore, we investigated whether STAT3 activation influenced expression of Thy-1/CD90 on LFs. We screened 14 primary LF cultures for Thy-1/CD90 expression and STAT3 activation and observed a statistically significant
that unlike β3 integrin, the inhibitory effect of STAT3 on Thy-1/CD90 expression is not transcriptionally regulated.

Inhibition of STAT3 Signaling in IPF LFs
Diminishes Responsiveness to Exogenous TGF-β1 and Reverses Resistance to Apoptosis

To more definitively determine the role of STAT3 signaling in IPF-derived fibroblasts, we infected IPF LFs with Ad-STATDN and analyzed cell responsiveness to recombinant human TGF-β1. Figure 8A demonstrates that in contrast to normal LFs, IPF LFs infected with Ad-STAT3DN

Figure 7. Basal levels of STAT3 activation is negatively correlated with Thy-1/CD90 cell surface expression on primary human LFs. A: Screening of 14 individual lines of LFs isolated from different compartments of the non-IPF lungs for Thy-1/CD90 cell surface expression determined by flow cytometry and basal levels of STAT3 activation revealed a negative correlation between the percentage of Thy-1/CD90 positive cells and the basal levels of pSTAT3 in the given cell population (P < 0.05). B: Expression of Thy-1/CD90 antigen on the cell surface of parental nontransfected control, stably STAT3C-transfected, and LacZ-transfected control LFs was determined by flow cytometry. Representative flow cytometry histograms from three independent experiments are shown. C: Effect of the STAT3 signaling inhibition by Ad-STAT3DN on basal and TGF-β1-mediated expression of Thy-1 mRNA in HFL-1 cells. Data are mean ± SE of the THY1 gene expression index determined in two independent experiments by quantitative RT-PCR performed in triplicates.

negative correlation (Figure 7A), suggesting a role for STAT3 signaling in the regulation of this molecule. Similar to that seen for β3 integrin expression, cells infected with STAT3C displayed a markedly reduced Thy-1/CD90 cell surface expression compared with noninfected or LacZ LF controls (Figure 7B). However, inhibition of STAT3 activity by Ad-STAT3DN infection did not significantly change Thy-1 mRNA expression levels in both TGF-β1–stimulated and control cultures (Figure 7C), suggesting

Figure 8. Blockade of STAT3 signaling in IPF LFs diminished cell responsiveness to exogenous TGF-β1 and apoptotic resistance. IPF LFs and control LFs were infected with Ad-STATDN and Ad-GFP as an infection control, and 24 hours later TGF-β1 was added to the cultures for 48 hours. α-SMA in total protein extracts was detected by immunoblotting. A: One representative immunoblot of four independent experiments is shown; total STAT3 protein is significantly elevated in Ad-STATDN IPF LFs and control LFs, confirming expression of the mutant protein in both cell lines. β-Tubulin was used as a loading control. B: Basal and TGF-β1–induced α-SMA protein in IPF LFs infected with Ad-STAT3DN and Ad-GFP as a control. Data are mean ± SE of percent changes of α-SMA/β-tubulin ratios. *P < 0.05 compared with Ad-GFP and Ad-STAT3DN without TGF-β1 stimulation. C: α-SMA gene expression in the same IPF LFs described in B, data are mean ± SE of the α-SMA gene expression index determined in two different primary IPF LF cultures by quantitative RT-PCR performed in triplicate. *P < 0.05 compared with Ad-GFP and Ad-STAT3DN without TGF-β1 stimulation. D: IPF LFs and control LFs were infected with Ad-STAT3DN and Ad-GFP; 24 hours after infection all cells were treated with staurosporine in serum-free medium to induce apoptosis. Total procaspase-3 and cleaved fragments of activated caspase-3 were detected by immunoblotting. Densitometry ratios of cleaved/total procaspase-3 were calculated and considered as a caspase-3 activation index. Graph data are mean ± SE of percent changes of caspase-3 activation calculated independently for control LFs and IPF LFs. *P < 0.05 compared with Ad-GFP and Ad-STAT3WT (n = 3).
displayed diminished responsiveness to TGF-β1, expressing significantly lower levels of α-SMA protein (Figure 8B) and mRNA (Figure 8C) after TGF-β1 stimulation compared with Ad-GFP infected controls. In contrast to normal LFs (Figures 4 and 5), inhibition of basal STAT3 signaling in IPF LFs did not significantly change constitutive α-SMA expression levels (Figure 8, B and C).

We have previously shown that IPF LFs are resistant to Fas-induced apoptosis in a STAT3-dependent manner. In the present study, we investigated the effect of STAT3 inhibition on IPF LF apoptosis induced by staurosporine, a broad-spectrum kinase inhibitor with a strong apoptotic capability mediated via the caspase-3–dependent pathway. As shown in Figure 8D, STAT3DN significantly enhanced staurosporine-induced caspase-3 activation in IPF LFs as detected by the increase of cleaved fragments of procaspase-3. Moreover, infection of LFs with Ad-STAT3WT, which drastically increased basal STAT3 activity in LFs (see Supplemental Figure S2 at http://ajp.amjpathol.org), inhibited caspase-3 activation induced by the staurosporine exposure in IPF LFs but not in normal LFs (Figure 8D), suggesting a different role for STAT3 signaling in apoptotic resistance in normal LFs and IPF-derived fibroblasts.

Discussion

We performed this study to examine whether STAT3-mediated signaling plays an important role in the pathogenesis of UIP/IPF by inducing aberrant activation and differentiation of fibroblasts and whether manipulation of this signaling pathway is a potential therapeutic target in IPF. We isolated a population of fibroblasts from the lungs of patients with UIP/IPF, which were characterized by constitutively high levels of pSTAT3. These cells displayed persistent morphologic and functional abnormalities in vitro, including reduced proliferation and expression of α-SMA, Thy-1/CD90, and α, β3 integrins but increased expression of fibronectin. Furthermore, we observed a large number of cells expressing pSTAT3 in UIP/IPF lungs, particularly in areas of dense fibrosis, but staining was not seen in the α-SMA–positive fibroblastic foci. Although these observations suggest STAT3 is involved in the development of this fibroblast phenotype, they do not show causality. To investigate this, we generated several in vitro models that allowed us to manipulate STAT3 signaling in primary LFs.

We show that forced induction of STAT3 signaling in LFs drives these cells toward an abnormal phenotype, which bears a striking resemblance with fibroblasts we isolated from UIP/IPF lung tissue. We observed an inhibitory effect of the STAT3-activating cytokines IL-6 and OSM on basal and TGF-β1–induced α-SMA expression, suggesting a regulatory effect on the differentiation of myofibroblasts. Going further, LFs infected with STAT3DN or treated with the STAT3 inhibitor STA-21 showed increased α-SMA expression and production of β3 integrin, both of which were dependent on TGF-β receptor I (TGFβRI)/ALK-5 signaling. Although neither constitutive nor cytokine-induced activation of STAT3 in LFs had any effect on collagen I expression or secretion, inhibition of STAT3 signaling in IPF LFs diminished both basal and TGF-β1–induced secretion of collagen I. Moreover, we found that blockade of STAT3-mediated signaling in IPF LFs significantly attenuated sensitivity to exogenous TGF-β1 and, in contrast to normal LFs, augmented staurosporine-induced apoptosis. Considering that STAT3 is an essential transcription factor for several facets of cell differentiation, proliferation, and apoptosis, these findings suggest that sustained and enhanced activation of STAT3 signaling is an important mechanism dysregulating normal reparative functions of resident fibroblasts and myofibroblasts in the human lung.

We have previously shown that a population of fibroblasts isolated from IPF lungs responds to IL-6 stimulation with blunted activation of STAT3. Although most of these cells were α-SMA positive, they displayed enhanced proliferative activity and resistance to Fas-induced apoptosis compared with non-IPF control fibroblasts. In the present study, we describe another population of fibroblasts isolated from a larger number of IPF lungs, characterized by significantly lower proliferative activity but normal STAT3 activation in response to IL-10 and gp130 cytokine stimulation. These cells are larger than LFs derived from non-IPF lungs and display diminished expression of α-SMA and two key adhesion molecules αv, β3 integrin and CD90/Thy-1 but increased expression of fibronectin. Similar to our findings, Ramos and associates reported on a population of fibroblasts from IPF lungs that exhibited slower growth rate and an increased rate of spontaneous apoptosis compared with control cells. Interestingly, Lim and colleagues reported enhanced levels of pSTAT3 in fibroblasts derived from keloids, but in contrast to our findings, these cells demonstrated significantly increased proliferative and synthetic activity compared with normal dermal fibroblasts. We and others have recently shown that normal primary human lung parenchyma–derived fibroblasts display classic myofibroblastic phenotype characterized by constitutively active TGF-β/Smad signaling and high levels of α-SMA expression, contractility, and proliferation. However, somewhat surprisingly, these myofibroblasts displayed decreased ECM synthetic capacity and lower responsiveness to exogenously added biologically active TGF-β1 compared with paired airway-derived cells, suggesting some self-limiting mechanisms for ECM synthesis in highly contractile α-SMA–expressing myofibroblasts. In support of this, ablation of α-SMA expression worsens the course of fibrosis in mouse kidney and lung. Alternatively, activation of STAT3 signaling might induce differentiation of resident LFs toward a stable and functionally distinct population of cells within IPF lung. Adding to this paradigm of fibroblast heterogeneity, the data in this article suggest that LFs might also be separated into two distinct subpopulations based on the level of STAT3 activation, with LF-pSTAT3high and LF-pSTAT3low cells consistently demonstrating the opposite phenotypic and functional responses. Thus, these observations, together with the findings that absence of Thy-1/CD90 expression confers a profibrotic phenotype to IPF LFs with enhanced responsiveness to TGF-β1,
support our hypothesis that STAT3-mediated suppression of constitutive α-SMA, αv β3 integrin, and Thy-1/CD90 expression is critically important for pathological phenotype of mesenchymal cells in UIP/IPF lung.

To explore this concept further, we infected normal LFs with constructs encoding wild-type STAT3 protein, STAT3C,26 or STAT3DN15 mutants and examined expression of several key parameters of activation and fibroblast/myofibroblast differentiation. We found that expression of STAT3C in normal LFs significantly reduced surface presentation of Thy-1/CD90. Furthermore, examination of a large number of different human primary fibroblast lines isolated from non-IPF lungs also revealed a negative correlation between the levels of pSTAT3 and surface expression of Thy-1/CD90. Interestingly, STAT3-modified Thy-1 on the cell surface without affecting the gene transcription. Thy-1 is a 25–37-kDa cell membrane–anchored protein implicated in many aspects of wound healing and fibrosis, although its precise roles are poorly defined.6,37,43,44 Differential expression of Thy-1/CD90 is suggested to be a reliable marker of the fibrosis because cells within fibroblastic foci are Thy-1(–), whereas normal lung fibroblasts are predominantly Thy-1(+).5,12,45 More recently, Zhou and colleagues demonstrated that the interaction of αv β3 integrin with Thy-1 is a critical element of myofibroblast contraction and latent TGF-β activation.38 Thus, the reduced expression of Thy-1/CD90 and αv β3 but enhanced αv β5 integrin expression mediated by STAT3 may disrupt normal integrin-mediated signaling, which in turn is likely responsible for the higher responsiveness of IPF LFs to TGF-β and other profibrotic factors.32

In line with previous observations, we also found that IPF LFs were deficient in β3 integrin and α-SMA expression, two critical elements of fibroblast/myofibroblast activation, differentiation, and TGF-β signaling.46,47 We have previously shown that αv β3 plays an important role in regulating myofibroblast functions, including cell responsiveness to TGF-β1 and communication with the ECM.7,36,48 Accordingly, we investigated whether STAT3 was involved in regulating αv β3 expression and found that STAT3 activation is associated with diminished β3-integrin gene expression and surface expression of αv β3 but not αv β5 integrins in LFs. In contrast, inhibition of endogenous STAT3 activity restored β3-integrin expression most dramatically in LF-pSTAT3H140. These two pieces of evidence strongly suggest a direct suppressive role of STAT3 on β3 integrin and, most likely, indirectly on latent TGF-β activation and, therefore, myofibroblast differentiation.36,38,48 Previously, αv β3 and αv β5 integrins have been demonstrated to display a different functional role in processes of cell adhesion and migration49 and binding capacity to TGFβRII.48 Both of these processes are relevant to the enhanced fibrogenic activity of IPF LFs. It has been shown that wound healing in β3-integrin knockout mice was accompanied by enhanced TGF-β signaling.50

Similar observations were made in transgenic mice with a kinase-deficient TGFβRII51 and in aortic tissues from patients with the Loeyls-Dietz syndrome associated with R495X mutation of TGFβRII.52 In addition, Pannu and associates have reported that enhanced collagen production and responsiveness of SSc-derived fibroblasts to TGF-β were associated with diminished cell surface expression of TGFβRII.53 Taking into consideration that αv β3 but not αv β5 is a functional partner of TGFβRII54 and in this study IPF LFs were characterized by a decreased β3-integrin expression and enhanced responsiveness to TGF-β1 stimulation, we speculate that exaggerated activation of STAT3 signaling in IPF fibroblasts is responsible for that phenomenon. Currently, a role for αv β3 and αv β5 integrins in UIP/IPF pathogenesis is unknown.54

Experiments with Ad-STAT3DN–infected or STA-21–exposed LFs show that even in fibroblasts with undetectable levels of pSTAT3 there is basal activity of this intracellular signaling pathway. Both of these inhibitory manipulations led to the increase of α-SMA and β3-integrin expression in normal LFs. Currently, we do not know which cytokines and growth factors are responsible for basal STAT3 activation in control LF and IPF LF cells; however, it is not likely confined to gp130 activation because we have shown that epidermal growth factor is a potent repressor of basal and TGF-β1–induced αv β3 expression in fibroblasts.36 Platelet-derived growth factor55 and other epithelium-derived growth factors together with IL-10,56 abundantly secreted by alternatively activated alveolar macrophages and signaling via STAT3-dependent pathways,57–59 are also potential candidates.

Cross talk between cytokines and growth factors is vital for the transition from wound closure to normal tissue function restoration or, if left uncontrolled, fibrosis.20,60 Emerging data describe a complex interaction between gp130/STAT3 and TGF-β/Smad in various pathological situations and suggest that although these molecules cooperate in the progression of some disease states, in others they are antagonistic.51–64 We tested the interaction between STAT3 and SMAD3 signaling on production of collagen I and α-SMA. As expected, TGF-β1 induced Smad3 phosphorylation and collagen I expression in LFs. Addition of IL-6 or OSM had no effect on collagen expression and secretion despite robust activation of STAT3 signaling, suggesting that STAT3 activity does not exert a significant effect on collagen I production in normal LFs. However, we observed robust inhibitory effect of STAT3DN on collagen I secretion in some IPF LF lines. Interestingly, this effect was only evident in IPF LF cultures at baseline and after TGF-β1 stimulation, suggesting a different and yet unknown mechanism of a STAT3 signaling inhibition on collagen I secretion in IPF-derived fibroblasts. Surprisingly, we found that activation of STAT3 signaling in normal LFs is associated with depression of TGF-β1–induced α-SMA expression. Taking into consideration a steady-state level of pSTAT3 induced by OSM in LFs, it is likely that STAT3 signaling was involved in the observed antagonistic effect of two cytokines on α-SMA expression. Indeed, blockade of STAT3 signaling noticeably attenuated the inhibitory effect of OSM on α-SMA expression. These results are in line with the recent findings by Stangou et al.65 who demonstrated that in rats with an experimental kidney disease, treatment with human IL-11 depressed TGF-β signaling and impaired expression of α-SMA. However, although the in-
hibitory effect of OSM appears to involve signaling through ALK-5, SMAD3 phosphorylation was not reduced by the OSM-induced STAT3 activation. The inhibition of ALK-5-mediated signaling, which is upstream of both SMAD-dependent and SMAD-independent pathways, strongly attenuated the increased α-SMA and β3-integrin expression after STAT3 inhibition. In keeping with a SMAD3-independent effect, SMAD7 expression was not modified by Ad-STAT3DN. In a previous study, we demonstrated that β3-integrin expression induced by TGF-β1 was insensitive to overexpression of SMAD7.27 Therefore, STAT3 inhibition is affecting β3-integrin and α-SMA expression in LFs by ALK-5-dependent but SMAD-independent mechanisms. The most likely candidate is p38MAPK-mediated signaling, which we and others have demonstrated plays a key role in TGF-β1-induced β3-integrin and α-SMA expression and ultimately for contractile function of myofibroblasts36,46,66 and which has been shown engaged in negative feedback inhibition of STAT3 activity.67,68 In contrast to normal LFs, STAT3DN significantly attenuated TGF-β1-induced α-SMA expression in IPF LFs, further supporting our hypothesis of an important role for the aberrant STAT3 signaling in a shaping of fibroblast phenotypes in UIP/IPF.

Altogether, these findings clearly suggest that fibroblasts with high STAT3 activity, which we found in abundance within the dense fibrotic areas of IPF lungs, have impaired expression of α-SMA at baseline, which it seems is paralleled by an elevated capacity to express ECM proteins and up-regulate TGF-β-mediated signaling, down-regulation of several key adhesion molecules, and growth inhibition. The reasons for the appearance of this fibroblast phenotype in IPF are unknown, although it is worth noting that active STAT3 signaling mediates several antia apoptotic mechanisms in fibroblasts, including premature senescence.27,69,70 However, the precise mechanism of STAT3-mediated resistance of human lung fibroblasts to apoptosis and its role in the pathogenesis of UIP/IPF is largely unknown.

We have previously identified several significant differences between non-IPF and IPF-derived fibroblasts in terms of apoptosis.30 Those IPF LF lines had lower levels of apoptosis at baseline and after combined gp130/FAS receptor activation in a STAT3-dependent manner. Similarly, modulation of STAT3 activity in embryonic mouse and human skin fibroblasts has been shown to significantly change their resistance to apoptosis through modulation of both extrinsic and intrinsic pathways of programmed cell death.27,69,70 Data from the current study support these earlier findings. Indeed, inhibition of basal STAT3 activity in IPF-derived fibroblasts sensitizes cells to staurosporine-induced apoptosis via caspase-3-dependent pathway, whereas constitutively active STAT3 signaling led to inhibition of caspase-3 activation. In contrast, modulating STAT3 activity had little effect on the staurosporine-induced caspase-3 activation in normal LFs. These data support our hypothesis that enhanced STAT3 activity in IPF fibroblasts might be responsible for their persistence and fibrogenic activity in the areas of dense fibrosis in UIP via inhibition of several proapoptotic pathways.

In summary, we describe for the first time a role for STAT3 in regulating of fibroblast phenotype and function in UIP/IPF. Taking into consideration that STAT3 is the main signaling pathway activated by several cytokines associated with pulmonary fibrosis, including the IL-6 family, IL-10, and receptor tyrosine kinase activators such as epidermal growth factor and platelet-derived growth factor, it is likely that by regulating myofibroblast function, persistent STAT3 activation may be one of the critical mechanisms in the pathogenesis of UIP/IPF. We believe that assessment of STAT3 signaling pathway activation in cells or tissue derived from pathological regions of the UIP/IPF lungs might serve as a phenotypic and prognostic marker of the disease.

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