Plasminogen Activator Inhibitor-1 Suppresses Profibrotic Responses in Fibroblasts from Fibrotic Lungs*  

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Background: The long term survival outcome of patients with IPF is bleak, with a paucity of effective treatments. Results: The changes in baseline PAI-1 expression regulate fibroblast activation and expansion in fibrotic lung diseases. Conclusion: Targeted restoration, rather than inhibition of PAI-1 in activated fibroblasts, mitigates fibrosis. Significance: This study defines a new role of PAI-1 in the pathogenesis of fibrosing lung diseases, including IPF.

Idiopathic pulmonary fibrosis (IPF) is a fatal lung disease characterized by progressive interstitial scarification. A hallmark morphological lesion is the accumulation of myofibroblasts or fibrotic lung fibroblasts (FL-fibroblasts) in areas called fibrotic foci. We previously demonstrated that the expression of both urokinase-type plasminogen activator (uPA) and the uPA receptor are elevated in FL-fibroblasts from the lungs of patients with IPF. FL-fibroblasts isolated from human IPF lungs and from mice with bleomycin-induced pulmonary fibrosis showed an increased rate of proliferation compared with normal lung fibroblasts (NL-fibroblasts) derived from histologically “normal” lung. Basal expression of plasminogen activator inhibitor-1 (PAI-1) in human and murine FL-fibroblasts was reduced, whereas collagen-I and α-smooth muscle actin were markedly elevated. Conversely, alveolar type II epithelial cells surrounding the fibrotic foci in situ, as well as those isolated from IPF lungs, showed increased activation of caspase-3 and PAI-1 with a parallel reduction in uPA expression. Transduction of an adenovirus PAI-1 cDNA construct (Ad-PAI-1) suppressed expression of uPA and collagen-I and attenuated proliferation in FL-fibroblasts. On the contrary, inhibition of basal PAI-1 in NL-fibroblasts increased collagen-I and α-smooth muscle actin. Fibroblasts isolated from PAI-1-deficient mice without lung injury also showed increased collagen-I and uPA. These changes were associated with increased Akt/phosphatase and tensin homolog proliferation/survival signals in FL-fibroblasts, which were reversed by transduction with Ad-PAI-1. This study defines a new role of PAI-1 in the control of fibroblast activation and expansion and its role in the pathogenesis of fibrosing lung disease and, in particular, IPF.

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive and devastating lung disease of unknown etiology. Present estimates of the disease incidence are 40–50 per 100,000 in the United States. The majority of patients present with advanced disease at the time of diagnosis, and the long term survival outcome of patients with IPF is dismal, with a median 5-year survival of only 20% (1). Anti-inflammatory therapy has been unsuccessful, and newly discovered pharmacotherapeutic options remain limited (2, 3). The pathogenesis of IPF is complex, but the preponderance of the current literature suggests that type II alveolar epithelial cell (ATII cell) injury/apoptosis and the expansion of the fibrotic lung (FL)-fibroblast population in IPF leads to progressive deposition of extracellular matrix (ECM) and progressive pulmonary fibrosis (4–8). FL-fibroblasts are highly contractile, proliferate readily, and contribute to wound closure with peripheral deposition of ECM (9). In the normal repair process, FL-fibroblasts undergo apoptosis, while other resident cells degrade the ECM (10). However, in the lungs of patients with IPF or other fibrotic disorders, FL-fibroblasts resist apoptosis and exhibit unrestricted proliferation with ultimate compromise of the normal alveolar architecture. Because FL-fibroblasts are prime effector cells (11), there is a strong rationale to more clearly define mechanisms by which these cells contribute to exuberant repair and pulmonary fibrosis. We used primary human FL-fibroblasts from patients with IPF (hFL-fibroblasts) and mice with bleomycin (BLM)-induced lung fibrosis (mFL-fibroblasts), lung tissues, and ATII cells to assess the contribution of plasminogen activator inhibitor-1 (PAI-1) to the fibrogenic response.

PAI-1 levels are significantly increased in lung tissues, BAL fluids, and the ATII cells of patients with diverse lung diseases such as acute lung injury, its most severe clinical counterpart.

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3 The abbreviations used are: IPF, idiopathic pulmonary fibrosis; FL, fibrotic fibroblast; BLM, bleomycin; PAI-1, plasminogen activator inhibitor-1; uPA, urokinase-type plasminogen activator; ATII, alveolar type II; NL, normal lung; α-SMA, α-smooth muscle actin; ECM, extracellular matrix; FL, fibrotic lung; h, human; m, mouse; IHC, immunohistochemical; Col-I, collagen-I; RT-qPCR, RT-quantitative PCR; rPAI-1, recombinant PAI-1; PTEN, phosphatase and tensin homolog; CT, computerized tomography; SP-C, surfactant protein C.
acute respiratory distress syndrome, or IPF, as well as in mice with cigarette smoke- and BLM-induced lung injuries (12, 13). On the contrary, PAI-1 suppression prevents lung inflammation, ATII cell senescence and apoptosis, and the development of lung fibrosis (12–15). However, recent reports indicate that PAI-1-deficient NL-fibroblasts resist cigarette smoke-induced replicative senescence (16), although PAI-1 deficiency aggravates kidney (17), pleural (18), and cardiac fibrosis (19). These findings challenge the well established concept that global expression of PAI-1 in all lung cells powerfully exerts an exclusively pro-fibrogenic effect (12, 15, 20). Based on our recent observations, we inferred that PAI-1-induced changes in ATII cell viability (12, 13, 15) could differ from those induced in FL-fibroblasts that contribute to the expanding population of mesenchymal cells in lungs undergoing fibrotic repair. In this study, we therefore sought to assess the role of PAI-1 in pulmonary fibrosis using FL-fibroblasts isolated from the lungs of human IPF patients and from mice with BLM-induced lung fibrosis. Our findings indicate that unlike ATII cells in the lungs of IPF patients or mice with BLM-induced lung injury, baseline PAI-1 levels are significantly suppressed in hFL-fibroblasts or mFL-fibroblasts, although expressions of the urokinase-type plasminogen activator (uPA), collagen-I (Col-I), and α-smooth muscle actin (α-SMA) are elevated. These changes are reversed by restoration of PAI-1 expression in FL-fibroblasts. The process involves parallel inhibition of Akt/PTEN cell proliferation/survival signaling, which is otherwise increased in FL-fibroblasts.

**EXPERIMENTAL PROCEDURES**

**IPF Tissues**—All studies that involve specimens derived from de-identified human lung tissues from surgical biopsies or harvested at the time of lung transplantation or autopsy of patients with IPF or control donors without known lung disease were approved by the ethics committees of all institutions involved, including University of Texas Health Science Center at Tyler. Lung tissues from The Mayo Clinic were used for isolation of ATII cells. Lung sections were obtained from the tissue archives of the Department of Pathology, University of Texas Health Science Center at Tyler, or were provided by the Lung Tissue Research Consortium. The clinical diagnoses of patients with IPF were established by history, physical examination, high resolution CT, pulmonary function tests, and diagnostic lung biopsy by clinicians with expertise in the area. In all IPF cases from which cells were used in this study, the pathological diagnosis was usual interstitial pneumonia based on the presence of restrictive lung disease in association with a compatible clinical presentation and lung pathology. Lung tissues were subjected to immunohistochemical (IHC) analysis to assess changes in PAI-1 and Ki-67 antigen levels. For co-localization studies, lung sections (5 μm) were deparaffinized using xylene, followed by rehydration and antigen retrieval. These sections were later incubated overnight with SP-C (Santa Cruz Biotechnology, Dallas), PAI-1, and cleaved caspase-3 (Abcam, Cambridge, MA) antibodies. Rabbit IgG was used as a negative control. The sections were then incubated with fluorochrome-conjugated secondary antibody. The stained sections were then examined with a confocal microscope (LSM510 META; Zeiss), and co-localization analysis was performed by software ZEN.

**Isolation of ATII Cells and Fibroblasts from Control and IPF Lungs and Analysis of uPA, PAI-1, and Col-I**—Total protein content in the homogenates from resected lung tissues of patients with IPF and control (“normal”) subjects was determined using BCA reagents and known amounts of bovine serum albumin as standards. Total proteins from the lung homogenates were analyzed for PAI-1, Col-I, and uPA expression by Western blotting. Total RNA isolated from control and IPF lung tissues were analyzed by real time quantitative PCR (RT-qPCR) for PAI-1, Col-I, and uPA mRNA. ATII cells were isolated from control and IPF lungs as we described elsewhere (21). The purities of isolated cell preparations were confirmed by staining for inclusion bodies using lithium carbonate prior to immunoblotting (21). Human normal lung (hNL)-fibroblasts and human fibrotic (IPF) lung (hFL)-fibroblasts were purchased from Dr. Cory Hogaboam, University of Michigan, Ann Arbor, or kindly provided by Dr. Ganesh Raghu, University of Washington, Seattle, or were isolated locally from control and IPF lungs using type I collagenase and hyaluronidase in Dulbecco’s modified Eagle’s medium (DMEM) as we described previously (22–26). Isolated lung fibroblasts were cultured in DMEM containing 10% fetal bovine serum at 37 °C and used within 4–7 passages of the initial isolation. Fibroblasts were switched to overnight serum deprivation before testing for changes in Col-I, uPA, and PAI-1 protein and mRNA by Western blotting and RT-qPCR, respectively.

**Analysis of Rate of PAI-1 mRNA Synthesis and PAI-1 mRNA Decay**—hNL- and hFL-fibroblasts were transduced with an adenovirus vector (Ad-vector) expressing chimeric PAI-1 5’promoter sequence (Ad-5’Pro) or 3’ UTR sequence (Ad-3’UTR)-luciferase reporter constructs. The fibroblasts exposed to Ad-vector incorporating a scrambled promoter sequence (Ad-scPro) and luciferase reporter constructs or empty Ad-vector (Ad-EV) or naive hNL- and hFL-fibroblasts were used as controls. The lysates were analyzed for changes in luciferase antigen and activity by both Western blotting and chemiluminescence assays, respectively, as we reported earlier (15). The rate of PAI-1 mRNA decay was determined by analyzing PAI-1 mRNA remaining at various time points after inhibiting ongoing transcription as described elsewhere (27).

**Murine Model of BLM-induced Established Pulmonary Fibrosis**—WT and PAI-1-deficient mice of C57BL-6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of University of Texas Health Science Center at Tyler. WT mice were exposed once to BLM (2 units/kg of body weight) in 50 μl of saline via intranasal instillation under anesthesia as we described earlier (12, 15). Control mice were exposed to 50 μl of saline. Twenty one days after inception of BLM-induced lung injury, stable pulmonary fibrosis was confirmed by small animal CT scanning and assessment of pulmonary function tests using a Flexivent system under anesthesia. In brief, anesthetized mice were intubated after swabbing with lidocaine; the vocal cords were visualized, and a 20GXI™ cannula was passed through the vocal cords and into the trachea. Mice were connected to the
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ventilator tubing and secured to the bed of the CT scanner. Prior to CT scanning, pulmonary function testing was done. CT images were obtained during a breath-hold. Lungs were later extracted from euthanized mice and subjected to total hydroxyproline content and IHC analysis. Fibroblasts were also isolated from the lungs of mice with established pulmonary fibrosis or from uninjured control mouse lungs as described previously (22, 24–26). After one passage, the fibroblasts were isolated and analyzed for Col-I, α-SMA, and PAI-1.

Transduction of Fibroblasts with Ad-PAI-1—FL-fibroblasts from the lungs of patients with IPF or mice with established pulmonary fibrosis as well as NL-fibroblasts were exposed to Ad-PAI-1. FL-fibroblasts treated with empty Ad-vector (Ad-EV) and adenoviral vector expressing recombinant β-galactosidase (Ad-LacZ) were used as controls. Seventy two hours after transduction, cells were analyzed for changes in Col-I, α-SMA, PAI-1, and uPA. hNL- and FL-fibroblasts cultured in culture dishes were treated with PAI-1 shRNA (1 × 10⁶) for 72 h to suppress baseline PAI-1 expression. NL- and FL-fibroblasts exposed to nonspecific control shRNA were used as controls.

Statistical Analysis—Difference between two groups was analyzed by Student’s t test and for multiple groups by one-way analysis of variance tests.

RESULTS

Differential Expression of PAI-1 in Proliferating hFL-fibroblasts Versus Apoptotic ATII Cells in IPF Lungs—Fibrotic foci with increased deposits of Col-I, vimentin, and other ECM proteins due to increased localization of hFL-fibroblasts typify lung sections of patients with IPF. However, IHC analysis using an anti-PAI-1 antibody showed relatively little PAI-1 staining in the fibrotic foci of IPF lung sections (Fig. 1A), although overall staining for PAI-1 antigen was markedly elevated throughout IPF lungs compared with NL tissues. In addition, intense staining for Ki-67 antigen within the fibrotic foci (Fig. 1B) suggests increased proliferation of hFL-fibroblasts in the IPF lungs. IHC analysis for PAI-1 and SP-C indicated that 93.4% of PAI-1-positive cells are SP-C positive, and 54.7% SP-C positive cells are positive for PAI-1 in IPF lung sections (Fig. 1C). Moreover, 70.8% of PAI-1-positive cells are also positive for active caspase-3, and 54.3% of caspase-3 positive cells are PAI-1 positive (Fig. 1D). This indicates increased PAI-1 expression in ATII cells bordering fibrotic foci, with the majority of these cells undergoing apoptosis.

Differential Expression of PAI-1 in ATII Cells, hFL-Fibroblasts, and hFL-Tissues—Fibrotic foci showed minimal staining for PAI-1, although PAI-1 has generally been reported to be increased in the lungs in evolving pulmonary fibrosis (12, 15, 20, 28, 29). We therefore sought to test the postulate that hFL-fibroblasts from IPF lungs express relatively low levels of PAI-1, although homogenates and injured ATII cells in IPF lung tissues exhibit increased PAI-1. To address this possibility, we isolated ATII cells and fibroblasts from the normal and IPF lungs and tested their baseline levels of PAI-1 and uPA. Analysis of lung homogenates (Fig. 2A) and ATII cells (Fig. 2B) from IPF lung tissues showed increased PAI-1 compared with their corresponding levels in control (normal) lung tissues. These changes were associated with increased activation of caspase-3 and consequent degradation of poly(ADP-ribose) polymerase, although uPA and SP-C were reduced, indicating ATII cell apoptosis. However, hFL-fibroblasts had significantly lower levels of PAI-1 and elevated uPA expression compared with the corresponding expression by hNL-fibroblasts (Fig. 2C). Because the literature (12, 15, 30–32) likewise implicates uPA as a determinant of the extent of pulmonary fibrosis after injury, we analyzed the same culture media for uPA and soluble Col-I. Interestingly, we found that hFL-fibroblasts (n = 6) with low baseline PAI-1 levels invariably showed higher uPA and Col-I expression.

We next analyzed IPF lungs for PAI-1, uPA, and Col-I mRNA and found that both PAI-1 and Col-I mRNA were increased, although uPA mRNA levels were relatively suppressed in hFL tissues (Fig. 2D). Because PAI-1 protein and mRNA expressions in hFL tissues were increased, whereas the IHC of fibrotic foci (Fig. 1A) and immunoblotting of hFL-fibroblasts (Fig. 2C) showed that basal PAI-1 protein levels were suppressed, we analyzed the RNA from hFL-fibroblasts for baseline PAI-1 and Col-I mRNA levels. Consistent with the expression of PAI-1 proteins, PAI-1 mRNA was significantly reduced, although baseline Col-I and uPA mRNAs were increased in hFL-fibroblasts compared with their levels in NL-fibroblasts (Fig. 2E).

TGF-β is the major cytokine that contributes to activation of fibroblasts and is elaborated in fibrotic lungs, including IPF lungs (33, 34). Furthermore, TGF-β augments PAI-1 expression via increased synthesis and stabilization of PAI-1 mRNA (27). We therefore next transduced hNL- and hFL-fibroblasts with Ad-vector containing luciferase reporter gene under the control of PAI-1 5′UTR promoter and 3′UTR sequences, and we analyzed the lysates to confirm whether the reduction in the rate of PAI-1 mRNA synthesis or the rate of PAI-1 mRNA decay contributed to the lower levels of PAI-1 expression in hFL-fibroblasts. We found that both baseline luciferase expression (Fig. 3A) and its activity (Fig. 3B) were increased in hNL-fibroblasts transduced with either 5′UTR promoter or 3′UTR sequences compared with their corresponding levels in hFL-fibroblasts. Analysis of the rate of PAI-1 mRNA decay (Fig. 3C) further confirmed the accelerated degradation of PAI-1 mRNA in hFL-fibroblasts. These findings confirm that suppression of PAI-1 mRNA synthesis and increased PAI-1 mRNA decay contribute to its reduced expression in FL-fibroblasts. Because TGF-β induces fibroblast activation and induces PAI-1 expression, we next treated hNL- and hFL-fibroblasts with TGF-β and assessed changes in PAI-1 and Col-I expression. We found that treatment of hNL-fibroblasts with TGF-β increased both PAI-1 and Col-I expression. However, TGF-β-treated hFL-fibroblasts showed muted induction of PAI-1 and failed to significantly increase Col-I expression from already elevated baseline levels (Fig. 3D).

Recent reports (12, 35, 36) attribute reduced viability of epithelial, endothelial, and mesenchymal cells in diverse lung diseases to a disproportionate increase in the expression of PAI-1. We (22) and others (7, 8, 37) have previously reported that the basal level of proliferation was significantly higher in hFL-fibroblasts than in hNL-fibroblasts, and IPF tissues showed increased Ki-67 antigen and reduced PAI-1 expression in fibrotic foci (Fig. 1). This suggests that viability of hFL-fibro-
blasts is increased due to inherent reduction of PAI-1 expression and may add to their overall expansion. These cells, with the injured epithelium, contribute to the overall increment of PAI-1 in lung homogenates of IPF patients. To next test PAI-1 expression in evolving pulmonary fibrosis, we subjected mice to BLM challenge. CT scanning (Fig. 4A) and pulmonary function tests (Fig. 4B) indicated that mice exposed to intranasal BLM had changes consistent with significant pulmonary fibrosis 21 days after BLM-induced lung injury. Analysis of lung homogenates for total hydroxyproline content (Fig. 4C) as well as IHC, H&E, and Mason’s Trichrome staining of lung sections (data not shown) confirmed pulmonary fibrosis in BLM mice. However, consistent with lung sections from patients with IPF, the fibrotic areas of lungs that were rich in vimentin and Col-I (data not shown) also showed minimal PAI-1 (Fig. 4D), despite increased overall PAI-1 levels in the lungs of mice with BLM-induced established lung fibrosis compared with control mice.

Analyses of lung homogenates of mice with established pulmonary fibrosis 21 days after BLM injury also displayed increased expression of Col-I and PAI-1 proteins, although the uPA level was reduced compared with mice treated with only saline (Fig. 5A). Consistent with mRNA from the lungs tissues of patients with IPF (Fig. 2D), PCR analysis of mRNA from the lungs of mice with BLM-induced lung injury indicated increased Col-I and PAI-1 mRNA expression (Fig. 5B). As was the case with hFL-fibroblasts, mFL-fibroblasts (passage 2) from

FIGURE 1. Histochemical analyses of IPF lung sections. Lung sections from patients with IPF and control or normal subjects (NL) were subjected to IHC analysis using anti-PAI-1 (A) and anti-Ki-67 (B) antibodies to assess their relative expression. Sections from IPF and NL tissues were exposed to anti-PAI-1 and anti-SP-C (C) or anti-PAI-1 and anti-active caspase-3 (D) antibodies and examined using confocal microscopy. These sections were later developed with fluorescently labeled secondary antibodies. Quantitation of the co-localization is shown at the bottom of the image. Arrowheads indicate fibrotic foci, and scale bar indicates 50 μm long in C and D. One representative example is shown (of n = 5 IPF lung specimens).
Regulation of Pulmonary Fibrosis by PAI-1

A. Homogenate

B. AT II cells

C. NL-Fibroblasts

D. Tissue

E. Fibroblasts

Protein Fold Change

Col-I
PAI-1
uPA
β-actin

Protein Fold Change

Col-I
PAI-1
uPA
β-actin

Protein Fold Change

Col-I
PAI-1
uPA
β-actin

Protein Fold Change

Col-I
PAI-1
uPA
β-actin

mRNA Fold Change

uPA
PAI-1
Col-I

mRNA Fold Change

uPA
PAI-1
Col-I

mRNA Fold Change

uPA
PAI-1
Col-I
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Inhibition of ECM Proteins by Forced Expression of PAI-1 in hFL-Fibroblasts—hFL-fibroblasts showed low basal levels of PAI-1 despite the elevated PAI-1 mRNA levels in the lungs of mice with BLM-induced lung fibrosis. These findings suggest a link between elevated expression of mFL-fibroblasts and increased production of ECM proteins in the lungs of mice with BLM-induced established lung fibrosis.

Role of PAI-1 mRNA transcription and stabilization in differential expression of PAI-1 in NL- and FL-fibroblasts. NL- and FL-fibroblasts were transduced with Ad-vector expressing a chimeric luciferase reporter gene under the PAI-1 5’UTR promoter (S’Pro) or a scrambled control promoter (scPro) or a 3’ UTR sequence (3’UTR). Naive NL- and FL-fibroblasts or those exposed to empty Ad-vector (Ad-EV) were used as additional controls. After 96 h, the lysates were analyzed for luciferase antigen by Western blotting (A) and luciferase activity chemiluminescence assays (B), respectively. C, total RNA was isolated from hNL- and hFL-fibroblasts between 0 and 24 h after inhibiting ongoing transcription using 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (20 μg/ml). An aliquot of isolated RNA was analyzed for PAI-1 mRNA and normalized against corresponding levels of β-actin mRNA. The rate of PAI-1 mRNA decay is illustrated as a line graph with transcript levels at 0 h considered 100%. Experiments were repeated at least twice, using different, randomly chosen samples from each group for analysis. D, NL- and FL-fibroblasts were treated with PBS or TGF-β (2 ng/ml) for 24 h at 37 °C. The condition media of hNL- and hFL-fibroblasts treated with PBS and TGF-β were analyzed for PAI-1 and soluble Col-I, and the lysates were tested for intracellular PAI-1 levels by Western blotting.

The mice with BLM-induced lung fibrosis showed increased Col-I and α-SMA compared with control mNL-fibroblasts (Fig. 5C). mFL-fibroblasts also showed markedly less PAI-1 and increased Col-I and uPA protein expression compared with basal levels in mNL-fibroblasts (Fig. 5D). Analyses for PAI-1 mRNA (Fig. 5E) indicated significant reductions, although expression of Col-I and uPA mRNAs were increased in mFL-fibroblasts. mFL-fibroblasts also exhibited an increased rate of proliferation compared with mNL-fibroblasts from control mice (Fig. 5F). These findings suggest a link between elevated proliferation of mFL-fibroblasts and increased production of ECM proteins in the lungs of mice with BLM-induced established lung fibrosis.

To determine whether inhibition of basal PAI-1 expression in fibroblasts alternatively contributes to excess production of Col-I and α-SMA, we transduced hNL- and hFL-fibroblasts with lentivirus vector expressing PAI-1 shRNA. The control
cells were treated with lentivirus expressing control nonspecific shRNA. Transduction of hNL-fibroblasts with shRNA inhibited PAI-1 protein without affecting FL-fibroblasts (Fig. 7A) and also inhibited mRNA in NL-fibroblasts (Fig. 7B) expression. Analysis of culture media for soluble Col-I and uPA indicated induction of hNL-fibroblasts following suppression of PAI-1 expression. Col-I and uPA mRNA expression was induced in PAI-1 shRNA-treated NL-fibroblasts. However, treatment of hFL-fibroblasts with PAI-1 shRNA failed to increase Col-I or uPA expression compared with elevated baseline levels, probably due to inherent low expression of PAI-1 by these cells. Next, we isolated fibroblasts from the lungs of PAI-1-deficient mice and analyzed baseline PAI-1 and Col-I expression. As shown in Fig. 7C, we found that fibroblasts isolated from the lungs of the deficient mice showed no PAI-1 as expected, but basal levels of Col-I were ~10 times higher than that of fibroblasts isolated from wild-type (WT) mice. These lung fibroblasts also showed increased basal uPA and α-SMA expression compared with fibroblasts from WT mice. Furthermore, transduction of two fibroblast lines isolated from two mice lacking PAI-1 expression with Ad-PAI-1 suppressed Col-I and α-SMA expression, although those treated with Ad-EV or Ad-LacZ showed elevated baseline Col-I, α-SMA, and uPA expression as in naive PAI-1-deficient cells (Fig. 7D). This indicates an intricate link between the activated phenotype of lung fibroblasts and the lack of basal PAI-1 expression.

Recent literature suggests that hyper-phosphorylation of Akt/PTEN in lung and muscle fibroblasts contributes to increased viability (37, 38). PAI-1 inhibits Akt phosphorylation in multiple cell types, including lung fibroblasts (16, 38). Therefore, we analyzed phosphorylation of Akt and PTEN in hFL-
Interestingly, we found that transduction of Ad-PAI-1 in hNL- or hFL-fibroblasts reduced phosphorylation of both Akt and PTEN proteins (Fig. 8A). Forced expression of PAI-1 in mFL-fibroblasts likewise reduced phosphorylation of Akt and PTEN (Fig. 8B). These findings suggest that inhibition of proliferation by restoring baseline PAI-1 expression in FL-fibroblasts limits their propensity to expand and to produce and deposit ECM proteins. The process involves suppression of Akt/PTEN proliferation and survival signals.
Regulation of Pulmonary Fibrosis by PAI-1

A

Human

|       | NL  | FL  |
|-------|-----|-----|
| Ad-LacZ |     |     |
| Ad-EV   |     |     |
| Ad-PAI-1 |    |     |
| Ad-LacZ |     |     |
| Ad-EV   |     |     |
| Ad-PAI-1 |    |     |
| α-SMA  |     |     |
| Col-I |     |     |
| P-Stat3 |     |     |
| Stat3 |     |     |
| PAI-1 cm |     |     |
| PAI-1 Lys- |   |     |
| uPA- |     |     |
| β-actin- |     |     |

B

Mouse

|       | NL  | FL  |
|-------|-----|-----|
| Ad-LacZ |     |     |
| Ad-EV   |     |     |
| Ad-PAI-1 |    |     |
| Ad-LacZ |     |     |
| Ad-EV   |     |     |
| Ad-PAI-1 |    |     |
| α-SMA  |     |     |
| Col-I |     |     |
| P-Stat3 |     |     |
| Stat3 |     |     |
| PAI-1 cm |     |     |
| PAI-1 Lys- |   |     |
| uPA- |     |     |
| β-actin- |     |     |

hFL-Fibroblasts

|       | Ad-EV | Ad-LacZ |
|-------|-------|---------|
|       |       |         |

Human CM

|       | Ad-LacZ | Ad-EV | Ad-PAI-1 |
|-------|---------|-------|----------|
|       |         |       |          |

Human Lys

|       | Ad-LacZ | Ad-EV | Ad-PAI-1 |
|-------|---------|-------|----------|
|       |         |       |          |

C

hFL-Fibroblasts

Collagen-I

|       | Saline | Ad-EV | Ad-PAI-1 |
|-------|--------|-------|----------|
| mRNA Fold change | 1.20 | 0.80 | 0.40 |
| p-value | <0.001 | <0.001 | <0.001 |

α-SMA

|       | Saline | Ad-EV | Ad-PAI-1 |
|-------|--------|-------|----------|
| mRNA Fold change | 1.20 | 0.80 | 0.40 |
| p-value | <0.001 | <0.001 | <0.001 |

D

mFL-Fibroblasts

Collagen-I

|       | Saline | Ad-EV | Ad-PAI-1 |
|-------|--------|-------|----------|
| mRNA Fold change | 1.20 | 0.80 | 0.40 |
| p-value | <0.001 | <0.001 | <0.001 |

α-SMA

|       | Saline | Ad-EV | Ad-PAI-1 |
|-------|--------|-------|----------|
| mRNA Fold change | 1.20 | 0.80 | 0.40 |
| p-value | <0.001 | <0.001 | <0.001 |

E

hFL-Fibroblasts

|       | None | Ad-EV | Ad-PAI-1 | rPAI-1 |
|-------|------|-------|----------|--------|
| Number of Cells X10⁴/mm² | 60 | 60 | 60 | 60 |
| p-value | <0.0001 |       |          |        |

F

mFL-Fibroblasts

|       | None | Ad-EV | Ad-PAI-1 | rPAI-1 |
|-------|------|-------|----------|--------|
| Number of Cells X10⁴/mm² | 80 | 80 | 80 | 80 |
| p-value | <0.0001 |       |          |        |
Increased expression of PAI-1 in lung tissues and ATII cells is often observed in the interstitial lung diseases and in models of pulmonary fibrosis (12, 15, 28, 29). We and others have attributed increased apoptosis or senescence of ATII cells and lung fibroblasts to inhibition of Akt survival signals and increased FIGURE 6. Inhibition of Col-I, α-SMA, and uPA by forced expression of PAI-1 in FL-fibroblasts. hNL-fibroblasts or FL-fibroblasts (n = 3) in culture dishes were treated with (1 × 10^5 cfu) PAI-1 shRNA or a nonspecific control shRNA in lentivirus vector. After 72 h, the conditioned media were analyzed for changes in PAI-1 and Col-I, and cell lysates were tested for α-SMA, uPA, PAI-1-phosphorylated and total Stat3 and β-actin by Western blotting. hFL-fibroblasts cultured in vitro were transduced with Ad-EV or Ad-PAI-1 or Ad-LacZ in culture dishes in vitro. After 72 h, the cells were analyzed for PAI-1, Col-I, α-SMA, uPA, phosphorylated/total Akt, and β-actin expression by Western blotting. Experiments were repeated at least three times.

DISCUSSION

Increased expression of PAI-1 in lung tissues and ATII cells is often observed in the interstitial lung diseases and in models of pulmonary fibrosis (12, 15, 28, 29). We and others have attributed increased apoptosis or senescence of ATII cells and lung fibroblasts to inhibition of Akt survival signals and increased
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PAI-1 levels in BLM- or cigarette smoke-induced lung injury (12, 13, 16, 27). On the contrary, genetic loss of PAI-1 causes increased Akt survival signaling and proliferation of muscle fibroblasts and muscle fibrosis in mdx dystrophic mice (38). These results suggest that alterations of PAI-1 can exert effects in the epithelium and fibroblasts alike, thereby influencing the progression of fibrosing lung injuries. ATII cell injury and apoptosis have been strongly implicated in the accumulation of FL-fibroblasts and dysregulated fibrotic repair in IPF and other interstitial lung diseases. Excessive FL-fibroblast activation, proliferation (22), migration (7, 8), release of pro-fibrotic cytokines (39), and resistance to apoptosis (40) all appear to contribute to the massive deposition of ECM and architectural distortion that occurs in IPF. A recent report by Larsson et al. (41) suggests that FL-fibroblasts constitute a major IPF parenchymal lung cell population.

Multiple studies suggest that PAI-1 potentiates pulmonary fibrosis based on the premise that PAI-1 is increased in the lungs after injury and either genetic deficiency or inhibition of PAI-1 in mice or rats protects them from lung injury and development of lung fibrosis (12, 15, 20, 28, 42–45). However, recent reports also indicate that mice deficient in PAI-1 expression are susceptible to cardiac, renal, and pleural fibrosis and muscular dystrophy (16–19, 38, 46–48). These diseases share many common pathophysiological features. Furthermore, PAI-1 deficiency promotes vein graft intimal hyperplasia by inducing endothelial and smooth muscle proliferation (47, 49, 50), PAI-1 deficiency circumvents replicative senescence through sustained activation of the PI3K-Akt pathway in primary lung fibroblasts (16). These observations contradict the recent report by Zhang et al. (51), who showed that expression of PAI-1 in fibroblasts isolated from the lungs of rats with BLM injury promotes proliferation and inhibits apoptosis through activation of the Akt survival pathway. In addition, a recent report by Chang et al. (23) with large variability in PAI-1 expression levels between four individual patient-derived cell lines showed that PAI-1 expression is slightly but significantly increased in the IPF fibroblasts, although our data clearly show that PAI-1 is reduced in FL-fibroblast populations harvested from the lungs of both IPF patients and BLM mice. The basis for the disparity is unclear to us but may be technical as the PAI-1 expression levels or phenotypes of isolated fibroblasts from BLM rats were not compared with control fibroblasts from uninjured rat lungs. The expression of PAI-1 in primary rat fibroblasts using pcDNA is likely much less efficient than the levels of expression by the adenoviral expression system we used. Chang et al. (23) attributed the variability in the expression of Col-I, α-SMA, phosphorylation of Akt, β-catenin, and PAI-1 between four individual patient-derived IPF fibroblast lines at least in part to the differences in the number of myofibroblasts in the isolates collected at various stages of diseases, heterogeneity of disease in IPF patients, genetic variability, and variable responses to culture conditions. Interestingly, Chang et al. (23) reported no significant difference in basal proliferation or survival of control versus IPF fibroblasts despite a slight but significant increase in PAI-1 expression in IPF fibroblasts, although Zhang et al. (51) found that PAI-1 increases proliferation and inhibits apoptosis in fibroblasts isolated from BLM-treated rats. Furthermore, the reported PAI-1 increments in IPF fibroblasts (23, 51) prompted us to systematically evaluate the effects of PAI-1 on pro-fibrogenic functional and phenotypic changes of fibroblasts versus ATII cells harvested from control and IPF lungs.

We found that the hFL-fibroblasts from the IPF lungs that expressed elevated baseline levels of Col-I and uPA also showed relatively reduced levels of PAI-1 versus hNL-fibroblasts. Consistent with prior reports (7, 8, 37), the basal rates of proliferation of hFL-fibroblasts are higher than in hNL-fibroblasts. Other groups have indicated that increased viability through hyper-phosphorylation of Akt survival signaling by hFL-fibroblasts contributes to excessive production of ECM proteins (52). Furthermore, inhibition of PI3K/Akt proliferation/survival signals that suppress fibrotic responses in IPF fibroblasts also induce PAI-1 expression (53). Our data using early passage hFL-fibroblasts derived from well characterized lung tissues clearly show that forced expression of PAI-1 using Ad-PAI-1 blocks the proliferative response and activation of Akt and Stat3. In addition, we found that mFL-fibroblasts from mice with established pulmonary fibrosis showed increased Col-I, α-SMA, and uPA expression and reduced baseline PAI-1 compared with corresponding mNL-fibroblasts. Consistent with PAI-1-deficient muscle fibroblasts (38), fibroblasts isolated from the lungs of mice lacking PAI-1 expression also showed increased baseline Col-I and α-SMA expression. Akt phosphorylation in PAI-1-deficient cells was also increased compared with that of WT fibroblasts despite the fact that PAI-1-deficient mice resist ATII cell apoptosis, lung injury, and pulmonary fibrosis caused by exposure to BLM or other agents (12, 13, 15, 21). PAI-1-deficient mice resist early alveolar epithelial injury, lung inflammation, and the development of pulmonary fibrosis despite elevated expression of Col-I, α-SMA, and uPA in their fibroblasts (this work) or increased expression of mesenchymal markers such as Col-I, α-SMA, and uPA in ATII cells (21). These findings and the inhibition of lung fibrosis by transplantation of uninjured ATII cells in mice with BLM- or silica-induced lung injury (54, 55) clearly suggest that ATII cell apoptosis plays a dominant role in subsequent development of lung fibrosis.
Similarly, inhibition of basal PAI-1 in hNL-fibroblasts, using shRNA, increased uPA and Col-I expression. These observations and the effects of PAI-1 up-regulation in either hFL-fibroblasts or mFL-fibroblasts extend our understanding of the common effects of increased PAI-1 on ATII cells, endothelial cells, and fibroblasts. We (12, 13, 15) and others (28) have demonstrated that mitigation of ATII cell apoptosis occurs via inhibition of PAI-1, which is otherwise induced during fibrosing lung injury. These observations support the contention that the well-established pro-fibrogenic effect of increased PAI-1-mediated ATII cell apoptosis can be prevented by the protection of ATII cells during lung injury using PAI-1 inhibitors (siRNA or chemical inhibitors), as reported by multiple laboratories, including ours (12, 13, 15, 19, 28, 56, 57). The role of PAI-1 in the regulation of fibroblast expansion, viability, and matrix deposition has been less well studied in the context of fibrosing lung injury, which is a basis for this report.

Our observations are further supported by the fact that the PI3K/Akt/GSK3β pathway regulates cyclin D1 kinase, which promotes cell proliferation (58). Akt phosphorylation is also increased in PAI-1-deficient cells, which is reversed by re-introduction of PAI-1 (16, 59). These findings clearly suggest that increased PI3K/Akt-mediated mitogenic signaling due to reduced PAI-1 expression in FL-fibroblasts or its absence in PAI-1-deficient fibroblasts support pro-fibrogenic responses. In addition, increased uPA expression occurs with suppression of PAI-1, which provides mitogenic signals with consequent uPA-uPA receptor autoregulatory feed-forward induction (60–62). In addition, interaction of PAI-1/vitronectin- and PAI-1/lipoprotein-related protein interactions have profound effects on cell adhesion and cell proliferation independent of anti-fibrinolytic activities (63–65). These could impact ECM production as in the case of ECM accumulation in experimental glomerulonephritis (66).

Consistent with our earlier report (34), we found that TGF-β induces PAI-1 in NL- and FL-fibroblasts, albeit to lesser extent in FL-fibroblasts. However, TGF-β significantly induces Col-I in NL-fibroblasts without affecting FL-fibroblasts, probably because of their elevated baseline expression of Col-I. These findings may relate to the known differences in expression of caveolin-1 between NL- and FL-fibroblasts. Caveolin-1 is markedly reduced in FL-fibroblasts isolated from both IPF and BLM-induced fibrotic lungs versus NL-fibroblasts (67). Our findings are also consistent with the observation that TGF-β fails to induce PAI-1 expression and SMAD2/3 phosphorylation in fibroblasts lacking caveolin-1 expression (68). This is also true with TGF-β-induced PAI-1 induction in hepatocytes that are caveolin-1-deficient (69). Furthermore, restoration of caveolin-1 rescues TGF-β-mediated induction of PAI-1 expression (70) and mice lacking caveolin-1 manifest severe fibrosis after BLM injury (71). Forced expression of caveolin-1 expression in FL-fibroblasts or in mice with BLM-induced lung injury mitigates pulmonary fibrosis (72). This probably involves rescue of PAI-1 expression because fibrotic lungs are rich in TGF-β. Finally, PAI-1 induces replicative senescence in fibroblasts such as MCF-7, IMR-90, and human stromal-derived breast fibroblasts (73–75). These findings are consistent with ours, showing that PAI-1 is decreased in FL-fibroblasts, which have acquired a highly proliferative/survival phenotype due to hyper-phosphorylation of Akt/PTEN.

In this study, we utilize multiple complementary but independent approaches and clearly demonstrate that PAI-1 inhibited the production of Col-I and other ECM proteins in FL-fibroblasts. The process involves PAI-1-mediated inhibition of FL-fibroblast proliferation through suppression of the Akt/PTEN proliferation/survival pathway. The effects are similar to those in ATII cells (12, 13) or fibroblasts (16), where inhibition of either uPA or uPA receptor augments PAI-1, thereby increasing cellular senescence and apoptosis. Interestingly, the viability of FL-fibroblasts was unaffected when treated with rPAI-1 protein, whereas elevated exogenous PAI-1 induces ATII cell apoptosis both in vitro and in vivo. The resistance of FL-fibroblasts to exogenous PAI-1 suggests that these cells are capable of flourishing in the PAI-1-rich milieu of injured lungs. The present observations offer a new paradigm by which PAI-1 may regulate coordinate changes in lung epithelial and fibroblast phenotypes in IPF. In IPF, our data indicate that ATII cells encasing fibrotic foci continuously die due to increased expression of PAI-1 by these cells. These wounds are then progressively populated by FL-fibroblasts that readily proliferate and secrete Col-I and other ECM, at least in part due to the lack of baseline PAI-1 expression. As the process evolves, progressive enlargement of fibrotic foci and fibrotic remodeling of the lung occurs. Inhibition of ECM deposition or proliferation of FL-fibroblasts by transduction with Ad-PAI-1 suggests that targeted restoration, rather than inhibition of PAI-1 expression in FL-fibroblasts, could mitigate fibrotic sequelae associated with IPF or other interstitial lung diseases.

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