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Forum Review

Oxidative Stress, Plasminogen Activator Inhibitor 1, and Lung Fibrosis

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ABSTRACT

Fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) in basement membranes and interstitial tissues, resulting from increased synthesis or decreased degradation of ECM or both. The plasminogen activator/plasmin system plays an important role in ECM degradation, whereas the plasminogen activator inhibitor 1 (PAI-1) is a physiologic inhibitor of plasminogen activators. PAI-1 expression is increased in the lung fibrotic diseases and in experimental fibrosis models. The deletion of the PAI-1 gene reduces, whereas the overexpression of PAI-1 enhances, the susceptibility of animals to lung fibrosis induced by different stimuli, indicating an important role of PAI-1 in the development of lung fibrosis. Many growth factors, including transforming growth factor beta (TGF-β) and tumor necrosis factor alpha (TNF-α), as well as other chemicals/agents, induce PAI-1 expression in cultured cells and *in vivo*. Reactive oxygen and nitrogen species (ROS/RNS) have been shown to mediate the induction of PAI-1 by many of these stimuli. This review summarizes some recent findings that help us to understand the role of PAI-1 in the development of lung fibrosis and ROS/RNS in the regulation of PAI-1 expression during fibrogenesis. *Antioxid. Redox Signal.* 10, 303–319.

INTRODUCTION

Fibrosis is a characteristic feature and terminal stage of many lung disorders, including idiopathic pulmonary fibrosis (IPF), hypersensitivity pneumonitis, drug- and radiation-induced lung injury, sarcoidosis, silicosis, asbestosis, cystic fibrosis (CF), and acute respiratory distress syndrome (ARDS). Despite the severe outcome, no efficacious treatment is known for these devastating fibrotic diseases because of a poor understanding of the complex pathologic processes. Fibrosis, characterized by excessive accumulation of extracellular matrix (ECM) in basement membranes and interstitial tissues, results from increased synthesis or decreased degradation of ECM (or both). Although intensive studies have been conducted, the molecular mechanism underlying the development of fibrosis is still not well understood. ECM degradation is mediated mainly by the matrix metalloproteinases (MMPs) and plasmin (4, 45, 129). Plasmin is converted from zymogen plasminogen by tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). The activities of tPA and uPA, under physiologic conditions, are controlled by plasminogen activator inhibitor 1 (PAI-1). Therefore, PAI-1 plays an important role in the regulation of ECM degradation. PAI-1 expression is increased in many fibrotic diseases and in experimental fibrosis models. Genetic modification of PAI-1 expression alters the sensitivity of animals to the fibrogenesis induced by various stimuli. These data suggest an important role of PAI-1 in the development of fibrosis, although the mechanism underlying the induction of PAI-1 during fibrogenesis and whereby PAI-1 promotes fibrosis remains to be further characterized. Emerging evidence indicates that reactive oxygen and nitrogen species (ROS/RNS) contribute importantly to the development of fibrosis under many pathologic conditions. The molecular mechanism whereby ROS/RNS mediate the fibrogenic effect, however, is largely unknown. This review focuses on the role

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of PAI-1 in the development of fibrosis and the redox regulation of PAI-1 gene expression during fibrogenesis.

PLASMINOGEN ACTIVATOR INHIBITOR-1 AND FIBROSIS

Plasminogen activation system and collagen degradation

ECM degradation is mediated mainly by two proteolytic systems, the fibrinolytic (plasminogen/plasmin) system and the MMP system. Although MMPs play a major role in the degradation of the ECM, increasing evidence indicates that plasmin is also important in ECM degradation. Plasmin can directly degrade various types of ECM proteins including fibronectin, laminin, proteoglycan, fibrin, denatured collagens, and type IV collagen (4, 5, 14, 59, 99, 148, 231). Most MMPs are synthesized and secreted as inactive proenzymes. The activities of MMPs are regulated at different levels, including gene expression, activation of proenzymes, and inhibition of active enzymes by tissue inhibitors of metalloproteinases (TIMPs) (106). Activation of promatrix metalloproteinases by sequential proteolysis of the propeptide that blocks the active-site cleft is regarded as one of the key levels of regulation of the activities for these proteinases. Although various agents/stimuli, including trypsin, ROS, and organomercurials, can activate MMPs *in vitro* (49, 80, 176, 177), a relatively well characterized mechanism for *in vivo* activation of MMP proenzymes involves the plasminogen activator–plasmin cascade (134, 143, 166, 175, 240). Therefore, in addition to degrading ECM proteins directly, plasmin can also activate MMPs, including MMP-2, MMP-3, MMP-9, MMP-12, and MMP-13 (106, 134, 143, 161, 166), which in turn degrade collagens and other ECM proteins.

Circulating plasminogen originates mainly from the liver, but many other tissues/organs, including kidney, lung, heart, brain, and spleen, also produce plasminogen locally (251). Inactive proenzyme plasminogen is converted to plasmin, a serine protease, by enzymatic cleavage of a 19-amino acid peptide, catalyzed by tPA and uPA. Plasminogen activation takes place in solutions, such as plasma, and on the cell surface. Many types of cells express plasminogen receptor on their surfaces, allowing localized activation of plasminogen (31). Binding to cell-surface receptors also substantially increases the rate of plasminogen activation, as compared with the reaction in solutions (58, 67, 159). In general, tPA is involved mainly in the activation of circulated plasminogen (fibrin clots), whereas uPA is involved in the activation of cell surface–bound plasminogen (67, 159). Under physiologic conditions, the activities of tPA and uPA are controlled by plasminogen activator inhibitors (PAIs), which inhibit plasminogen activator activity and therefore the activation of plasminogen, leading to ECM accumulation (Fig. 1).

Three PAIs have been identified, with type 1 plasminogen activator inhibitor (PAI-1) being the primary physiologic inhibitor of tPA and uPA *in vivo* (51). PAI-1 is a single-chain glycoprotein with a molecular mass of 50,000 Da, and it belongs to the serpin gene family (51). PAI-1 reacts very rapidly with single-chain and two-chain t-PA as well as with two-chain u-PA, with a second-order inhibition rate constant of 10^7 M/sec, but it does not react with single-chain u-PA (123). In addition to inhibiting t-PA/u-PA activity directly, PAI-1 can also block t-PA–mediated clot lysis by binding to fibrin and can inactivate u-PA *via* internalizing the u-PA and u-PA receptor (u-PAR) complex (43, 171, 196). PAI-1 is synthesized and secreted as an active form, which is unstable in solution and spontaneously converts into the inactive (latent) form with a half-life ~1–2 h at 37°C (136). Almost all of active PAI-1 in circulation binds to vitronectin, which extends its half-life by twofold to 10-fold. By binding to vitronectin, PAI-1 competes with u-PAR–dependent or integrin-dependent binding of cells to the ECM. Thereby, PAI-1 also plays an important role in cell adhesion and/or migration *via* a mechanism independent of its antiproteolytic activity (53, 135).

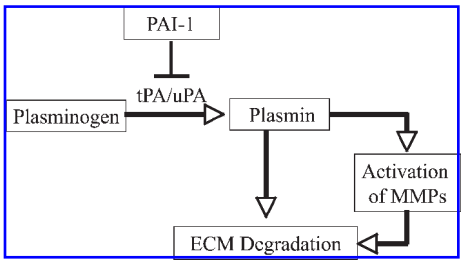

FIG. 1. Plasminogen activation system and extracellular matrix (ECM) degradation. Plasminogen is converted to plasmin by tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Plasmin then degrades ECM components directly or indirectly by activating matrix metalloproteinases (MMPs). Under physiologic conditions, the activities of tPA and uPA are controlled by plasminogen activator inhibitor 1 (PAI-1).

Increased PAI-1 expression in human lung fibrotic diseases

Fibrosis is a terminal stage of many lung disorders including IPF, hypersensitivity pneumonitis, drug- and radiation-induced lung injury, sarcoidosis, silicosis, asbestosis, CF, and ARDS. It has been reported that the fibrinolytic activity is decreased in broncheoalveolar lavage fluid (BALF) from patients with ARDS (13, 83, 100), IPF or interstitial lung diseases (33, 75, 84, 121), and sarcoidosis (33, 89). Such decreased fibrinolytic activity is associated with an increase in PAI-1 expression (75, 83, 121), suggesting an important role of PAI-1 in the development of the lung fibrotic diseases (13, 83, 84, 100).

IPF is a progressive and lethal fibrotic lung disease with unknown etiology and poor survival. It was speculated in the past that lung fibrosis results from an unremitting inflammatory response to an exogenous insult, which led to fibroblast activation/proliferation and eventually culminated in progressive fibrosis. Therefore, antiinflammatory agents alone or in combination with cytotoxic drugs have been used in clinics as a standard therapeutic regimen for the treatment of IPF. However, little evidence indicates that these agents alter the natural history of the disease or improve survival of the patients, suggesting that inflammation may not play a major role in the development of pulmonary fibrosis. Nevertheless, although the

mechanism underlying the development of IPF is unclear, emerging evidence indicates that increased expression of PAI-1 may contribute importantly to the pathogenesis of IPF. Nakstad *et al*. (167) reported that plasminogen activator activity was significantly lower, associated with high levels of antifibrinolytic activity, in IPF patients as compared with controls, suggesting that an imbalance between fibrinolytic and antifibrinolytic systems plays a role in the pathogenesis of IPF. Kotani *et al*. (121) also reported that PAI-1 levels in BAL supernatant fluids and PAI-2 levels in BAL cell lysates were significantly higher in IPF patients than those in normal subjects. These observations further suggest a critical role of PAI-1 in the development of IPF.

Several polymorphisms in the PAI-1 gene promoter, including the deletion/insertion polymorphism (4G/5G), have been reported and are associated with increased plasma levels of PAI-1 (152). *In vitro* experiments have shown that the 4G allele produces 6 times more PAI-1 RNA than the 5G allele in response to IL-1b (46). Individuals homozygous for the 4G allele have higher basal and inducible concentrations of PAI-1 than do those with one or two copies of the 5G allele (46). Interestingly, Kim *et al*. (116) reported that the patients with idiopathic interstitial pneumonia were more likely than the control population to have the promoter genotype of 4G/4G. These data provide direct evidence that IPF is genetically linked to high levels of PAI-1.

Sarcoidosis is a granulomatous disease associated with inflammation. Ninety percent of sarcoidosis cases are found in the lungs and, in many cases, will progress to pulmonary fibrosis. Chapman (33) reported that procoagulant activity in the macrophages extracted by lavage from patients with sarcoidosis was increased as compared with those from controls. Hasday *et al*. (89) further showed that mean procoagulant activity in the sarcoidosis group was significantly elevated, whereas plasminogen activator activity tended to be lower as compared with a control group. Therefore, it was concluded that in pulmonary sarcoidosis, abnormal expression of procoagulant and plasminogen activator activities in alveolar fluid may favor accumulation of fibrin matrix at inflammatory foci (89).

Cystic fibrosis is a genetic disease caused by mutations of the CF transmembrane conductance regulator (CFTR) gene (62, 239), which leads to a defect in CFTR proteins, a cAMP-dependent chloride channel. The major clinical problems associated with CFTR gene mutations are mucus accumulation, *Pseudomonas aeruginosa* infection, and chronic inflammation, with a consequence of remodeling and derangement of lung structure. Although the CFTR defect plays a dominant role in the development of CF, other factors may also contribute to the pathogenesis of the disease. Klinger *et al*. (118) reported that the PAI-1 gene, located at region q21.3-q22 of chromosome 7, is genetically linked with CF (118). Xiao *et al*. (241), furthermore showed that the PAI-1 level was elevated in induced sputum in patients with CF, which negatively correlated with pulmonary function. These observations indicate that PAI-1 may play a role in CF pathology.

Acute respiratory distress syndrome (ARDS) arises from a variety of local and systemic insults and is a major cause of acute respiratory failure. ARDS confers a high morbidity rate, in part due to the excessive deposition of collagen during the development of pulmonary fibrosis in the late-phase ARDS healing response. Fibrin deposition in the airspaces and lung microvasculature in ARDS results from the activation of the coagulation cascade and the impairment of fibrinolysis. Idell *et al*. (101) reported that procoagulant activity was increased in BAL and plasma 3 days after the onset of ARDS, whereas fibrinolytic activity was undetectable in BAL at 3 days after ARDS and remained depressed for up to 14 days. Such depressed fibrolytic activity was accompanied by an increase in PAI-1 activity (101). It has also been reported that alveolar PAI-1 antigen levels were more than 5 times higher in the patients with aspiration pneumonitis who progressed to ARDS than in those with uncomplicated aspiration pneumonitis, although plasma levels of PAI-1 antigen were not significantly different between the two groups (61). Several types of lung cells, including alveolar macrophages, endothelial cell, and epithelial cells, produce PAI-1 or PAs or both. Unstimulated alveolar macrophages usually express PA and are profibrinolytic; however, when stimulated, alveolar macrophages increased PAI-1 production and become antifibrolytic (34, 35). Chapman *et al*. (35) reported that alveolar macrophages from ADRS patients have increased PAI-1 mRNA, whereas Grau *et al*. (81) reported that lung microvascular endothelial cells isolated from ADRS patients expressed more PAI-1 than did controls and had a lower fibrinolytic potential, as measured by the PA/PAI-1 ratio. All these observations suggest that increased PAI-1 expression or decreased PA activity or both contribute importantly to the pathogenesis of ARDS.

Small-airway remodeling is a pathologic feature of asthma and contributes significantly to airflow obstruction. One of the major components of airway remodeling is excessive deposition of ECM proteins in bronchiolar walls (subepithelial fibrosis). Cho *et al*. (39) reported that mast cells from a patient with an asthma attack produced an increased amount of PAI-1. Buckova *et al*. (24) further showed that the 4G/5G polymorphism of the PAI-1 promoter is associated with an increased risk of asthma in the Czech population. These observations suggest a potential role of PAI-1 in asthmatic airway pathology (24, 39, 95).

PAI-1 and lung fibrosis: animal models

The central role of PAI-1 in the development of fibrosis has been well documented in several animal models. Bleomycin is a chemotherapy drug used for the treatment of some types of cancers. One of side effects associated with bleomycin treatment is lung fibrosis. The bleomycin-induced lung fibrosis model, therefore, has been widely used to study the pathogenesis of pulmonary fibrosis as well as the antifibrotic effects of potential therapeutic agents. It has been reported that intratracheal instillation of bleomycin to mice or rats increased PAI-1 activity in BALF and PAI-1 mRNA in the lung tissue, which was associated with a decrease in uPA activity (178, 253). It has also been shown that mice with homozygous deletion of PAI-1 were relatively protected from bleomycin-induced pulmonary fibrosis, whereas overexpression of PAI-1 enhanced bleomycin-induced lung fibrosis (40, 60, 90). Importantly, Hattori (90) showed that tranexamic acid, a plasminogen activation inhibitor, reversed the accelerated fibrin clearance observed in PAI-1–deficient mice, suggesting that increased plasminogen activation due to PAI-1 deficiency underlies the accelerated fibrin clearance observed in PAI-1–deficient mice. To fur-

ther test the hypothesis that PAI-1 deficiency limits scarring through unopposed plasminogen activation, a tetracycline-inducible uPA transgenic mouse model was established (213). After doxycycline administration, these transgenic animals expressed increased levels of uPA in their bronchoalveolar lavage fluid and reduced lung collagen accumulation as well as mortality as compared with control mice after bleomycin treatment (213). Swaisgood *et al*. (222) further showed that bleomycin-treated mice deficient for plasminogen or uPA had enhanced pulmonary fibrosis as compared with wild-type mice. By using adenovirus-mediated gene-transfer technique, Sisson *et al*. (214) showed that administration of human uPA–expressing adenovirus significantly reduced the amount of lung hydroxyproline and attenuated the bleomycin-induced lung collagen deposition. Taken together, the data suggest that the plasminogen system plays an essential role in ECM degradation and that increased PAI-1 expression promotes ECM deposition in bleomycin-treated mice probably by inhibiting plasminogen activation.

A potential role of PAI-1 in the development of lung fibrosis has also been studied in lung-fibrosis models induced by other stimuli. Silicosis is an occupational lung disease caused by inhalation of crystalline silica dust and features inflammation and scarring in the form of nodular lesions in the upper lobes of the lungs. Lardot *et al*. (128) reported that a single intratracheal administration of a fibrosing dose of crystalline silica in mice increased PAI-1 activity and protein levels in macrophages and neutrophils from BALF, associated with an induction of PAI-1 and PAI-2 mRNA in lung tissue. Sustained upregulation of PAI-1 and PAI- 2 mRNAs was still noted in lung tissue of these animals 1 month after silica treatment. These findings support the critical role of PAIs in the lung-remodeling process induced by silica. In an asthma model, Oh *et al*. (172) demonstrated that collagen deposition was twofold less, fibrin deposition was fourfold less, and MMP-9 activity was threefold higher in PAI-1 knockout mice than in wild-type mice after ovalbumin challenge, although the degree of airway inflammation was similar between PAI-1 knockout and wild-type mice. These results confirm a critical role of PAI-1 in ECM deposition in the airways of asthmatic patients (24, 39, 95).

Potential mechanisms whereby PAI-1 promotes ECM deposition

Although controversy still exists, three potential mechanisms whereby PAI-1 promotes ECM deposition have been proposed. The first and the most popular hypothesis is that PAI-1 promotes ECM deposition by inhibiting the activities of tPA and uPA, which leads to an inhibition of plasminogen activation and thus a decrease in ECM degradation. The observations from human diseases (13, 24, 33, 39, 46, 61, 75, 83, 84, 95, 100, 101, 116, 118, 121, 167, 242) and from experimental fibrosis models (40, 60, 90, 128, 172, 178, 253) strongly suggest that the abnormal induction of PAI-1 during tissue repair leads to the deposition of ECM by decreasing PA activity and the extent of plasmin formed; thus, the degradation of the ECM. In previous studies, we showed that transforming growth factor-beta (TGF-β), a most potent and ubiquitous profibrogenic cytokine, induced PAI-1 expression and inhibited the activities of tPA and plasmin as well as collagen degradation in cultured murine embryo fibroblasts (NIH3T3 cells) (234). Blockade of cell-surface binding of plasminogen/plasminogen activation with tranexamic acid or inhibition of plasmin activity with aprotinin significantly reduced the basal level of collagen degradation, both in the presence and absence of exogenous plasminogen (235). These data provide direct evidence that the plasminogen/plasmin system plays a critical role in collagen degradation and suggest that TGF-β inhibits collagen degradation probably by inducing PAI-1 expression and thus inhibiting plasminogen activation (235).

In addition to inhibiting ECM degradation, increasing evidence suggests other possible scenarios for PAI-1 effects. Matsuo (154) reported that at least four fibrogenic pathways were differentially expressed in PAI-1–overexpressing mice: (a) interstitial macrophage recruitment was more intense; (b) more interstitial myofibroblasts were found; (c) TGF-β and collagen I mRNA expression was higher; and (d) uPA activity was lower in PAI-1–overexpressing mice than in wild type mice (154). These results indicate that, in addition to potentially decreased fibrinolytic activity (decreased uPA activity), other mechanisms also contribute to the development of fibrosis observed in these PAI-1–overexpressing mice. As PAI-1 has been shown to regulate the adhesion and migration of a variety of cells *in vitro* and *in vivo* (6, 32, 51), it has been proposed that the elevated PAI-1 may promote ECM deposition by stimulating the migration of inflammatory or collagen-producing cells or both into the damaged tissue (140). Krag *et al*. (122) reported that although PAI-1 deficiency attenuated TGF-β–induced mesangial expansion, collagen accumulation, and basement-membrane thickening, no difference in protease activity was found between PAI-1–deficient and nondeficient mice. These data further support the notion that PAI-1 may promote ECM deposition through other mechanisms than inhibiting protease activity.

The third potential mechanism whereby PAI-1 promotes ECM deposition highlights the role of the plasminogen system in the release/activation of the antifibrotic growth factor, hepatocyte growth factor (HGF), from its sequestered sites, the extracellular matrix (69, 70, 91, 155, 160). Mizuno *et al*. (160) reported that administration of recombinant HGF to bleomycin-treated mice increased lung MMP activities, induced myofibroblast apoptosis, and stimulated ECM degradation, whereas injection with MMI270, a broad-spectrum MMP inhibitor, blocked HGF-induced MMP activation and myofibroblast apoptosis. These data suggest that HGF is a potent antifibrotic agent that blocks bleomycin-induced lung fibrosis by activating MMPs. Matsuoka *et al*. (155) further showed that plasminogen addition to NIH3T3 cells or mouse lung fibroblasts increased conversion of pro-HGF to its active form. They also showed that the plasminogen effect was lost when lung fibroblasts from uPA-deficient mice were used, and was increased by fibroblasts from PAI-1–deficient mice (155), indicating that release of ECM-bound HGF by NIH3T3 cells and mouse lung fibroblasts is plasminogen dependent (155). Most interestingly, it was reported that HGF protein levels in BALF from PAI-1 knockout mice were higher than those from wild-type mice after bleomycin administration (91). Blocking plasminogen activation with tranexanic acid reversed such an increase in HGF in the BALF from PAI-1 knockout mice, whereas administration of an anti-HGF neutralizing antibody markedly increased collagen accumulation in the lungs of bleomycin-treated PAI-

1 knockout mice (91). These results strongly suggest that, in addition to inhibiting tPA/uPA and ECM degradation, PAI-1 may promote ECM deposition by blocking HGF release/activation. A schematic diagram elucidating the potential mechanisms whereby PAI-1 promotes ECM deposition is presented in Fig. 2.

OXIDATIVE STRESS AND LUNG FIBROSIS

Fibrogenesis is a complex process and involves different types of cells as well as various cytokines, chemokines, and growth factors. Fibrogenesis occurs in most organs and can be induced by different stimuli, suggesting that common pathways or mechanisms may mediate this response (140). Although intensive studies have been conducted in the past decade, the molecular mechanism underlying this complex pathologic process is still not well understood. Oxidative stress occurs when exposure to oxidizing agents such as ozone and nitrogen dioxide, two important environmental pollutants, or the production of ROS/RNS is increased, or when the antioxidant capacity is decreased, or with a combination of these. Increasing evidence indicates that ROS/RNS play important roles in the development of fibrosis under various pathologic conditions such as liver fibrosis/cirrhosis caused by virus infection or alcohol consumption (8, 139, 182, 184, 186, 192, 212, 243), renal fibrosis associated with diabetics, hypertension, urinary tract obstruction (1, 55, 126, 168), and lung fibrosis (25, 42, 56, 72, 77, 150, 181, 185, 191, 194, 195, 199, 217) [for review see (117, 193)].

Increased oxidant production and oxidative damage in lung fibrotic diseases

The lung is a major target for oxidant damage because of its direct exposure to the atmosphere. Accumulating evidence suggests that oxidants generated endogenously or inhaled from the environment play an essential role in the pathogenesis of various pulmonary fibrotic disorders such as IPF, drug- and radiation-induced lung fibrosis, silicosis, asbestosis, CF, and ARDS (25, 42, 56, 72, 77, 117, 150, 181, 185, 187, 191, 193–195, 198–200, 217). It has been reported that inflammatory cells in BALF from IPF patients release increased amounts of reactive oxygen and nitrogen species (28, 191, 219, 220). Lipid and protein oxidation products, such as 8-isoprostane, have also been identified in the BALF or lung tissue in IPF patients (150, 163, 187). In addition to increased production of oxidants, numerous studies have shown that the levels of antioxidants including glutathione (GSH) (9, 10, 19, 21, 157, 162, 194) and the enzymes involved in the antioxidant defense such as MnSOD, catalase, and thioredoxin (127, 183, 228) are decreased in the lung-lining fluid of IPF patients. Increased oxidant production (56, 187, 195, 199, 200, 217) and decreased antioxidant capacity (30, 47, 181, 200, 201) have also been well documented in the lung tissue or BALF in CF patients. Such an imbalance between oxidants and antioxidants is most likely to contribute to the pathogenesis of IPF and CF, as antioxidant treatment has been shown to be able to improve the clinical manifestations (15, 52, 82, 88, 230).

Silicosis. Silicosis, one of the oldest occupational diseases, still kills thousands of people every year world-wide. It is an incurable lung disease caused by inhalation of dust containing free crystalline silica, which causes inflammation and scarring of the lung tissue. Exposure to particulate silica causes a persistent inflammation sustained by the release of oxidants in the alveolar space. ROS, which include hydroxyl radical, superoxide anion, hydrogen peroxide, and singlet oxygen, are generated not only at the silica particle surface but also by phagocytic cells attempting to digest the silica particle (16, 17, 72, 120, 233). The importance of ROS/RNS in silica-induced fibrosis has been well documented by studying the temporal relation between these events and by altering the fibrotic response with antioxidants. Porter *et al*. (188) reported that silica exposure induced pulmonary inflammation and damage in the rat lung tissue, which progressed to lung fibrosis, even after silica exposure ended. They further showed that silica-exposed rat lungs were in a state of oxidative stress and that silica-induced pulmonary NO and ROS production persisted even after silica exposure ended and the lung silica burden declined (189). These results suggest that ROS/RNS generated by silica itself or released by activated macrophages are responsible for the development of fibrosis in these silica-exposed rats (189). Gossart *et al*. (78) demonstrated that intratracheal instillation of silica in rats led to fibrosis characterized by cellular interstitial infiltrates with granulomas. Alveolar macrophages isolated from these rats showed an early and continuous increase in ROS production as well as TNF-α expression (78). Pretreatment of rats with a free radical scavenger, *N*-tert-butyl-α-phenylnitron, reversed lung histopathologic changes and decreased ROS production and TNF-α expression (78). Srivastava *et al*. (216) further showed that silica exposure resulted in lung inflammation, macrophage apoptosis, and significantly larger and more numerous silicotic lesions in wild-type mice than in inducible nitric oxide synthase (iNOS)–knockout mice, suggesting a role of NO in silica-induced lung injury. Taken together, these observations indicate that ROS/RNS mediate silica-induced lung fibrosis.

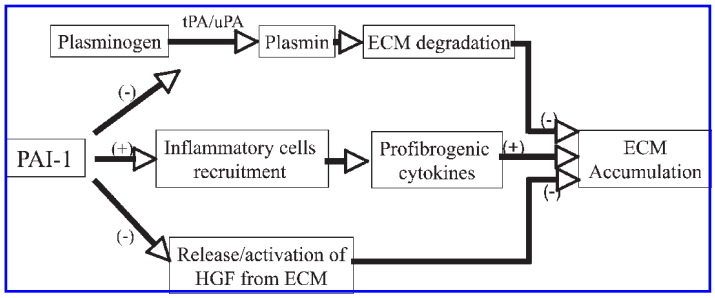

FIG. 2. Potential mechanisms whereby PAI-1 promotes ECM deposition. PAI-1 promotes ECM deposition by (a) inhibiting tPA and uPA activities and thereby plasminogen activation and ECM degradation; (b) recruiting inflammatory cells to the sites and therefore increasing profibrogenic cytokines; and (c) suppressing the release of antifibrogenic growth factors from the sequestered sites on the ECM.

Radiation-induced lung fibrosis. Progressive irreversible fibrosis is one of the most clinically significant sequences of ionizing radiation. Three successive clinical and histopathologic phases can be distinguished for radiation-induced fibrosis: the prefibrotic inflammatory phase, the constitutive fibrotic cellular phase, and the matrix densification/remodeling phase. Growing evidence indicates that ROS play a key role in radiation-induced lung fibrosis, although the molecular mechanism whereby ROS mediate fibrogenic effects of radiation is not well understood (12, 50, 63, 64, 130, 147, 197, 236). Hydroxyl radicals are generated by ionizing radiation, either directly by oxidation of water, or indirectly by the formation of secondary ROS, which can be subsequently converted to hydroxyl radicals by further reduction by metabolic processes in the cell. Therefore, secondary radiation injury is influenced by the cellular antioxidant status and the activating mechanisms. Numerous studies have shown that antioxidants reduce the radiation-induced fibrotic disorders, both in clinical practice and in animal experiments (50, 63, 64, 130, 147, 236), further suggesting an important role of ROS in radiation-induced lung fibrosis.

Bleomycin-induced lung fibrosis. Lung fibrosis is one of the major side effects caused by bleomycin, a chemotherapy drug used for the cancer treatment. ROS/RNS have been shown to play important roles in bleomycin-induced lung fibrosis. Inghilleri *et al.* (102) reported that bleomycin treatment increased ROS production in both phagocytes and in type II alveolar epithelial cells in rats. Manoury *et al.* (151) further showed that BAL cells from bleomycin-treated wild-type mice had enhanced ROS production, whereas no increase was observed with BAL cells from the mice deficient in $p47^{phox}$, a component of NADPH oxidase. Most important, no collagen deposition was found in the lungs of the $p47^{phox}$-knockout mice after bleomycin treatment, suggesting that NADPH-oxidase–derived ROS are essential to the development of pulmonary fibrosis in bleomycin-treated mice. Extracellular superoxide dismutase (EC-SOD) is highly expressed in the ECM of the lung. Localization of EC-SOD to the matrix of the lung may protect against oxidative tissue damage that leads to pulmonary fibrosis. Fattman *et al.* (66) showed that the severity of lung fibrosis after bleomycin treatment was significantly increased in EC-SOD null mice as compared with wild-type mice.

The importance of ROS/RNS in the development of bleomycin-induced lung fibrosis has also been well elucidated in numerous studies with antioxidants. Chemicals with antioxidant properties or the enzymes involved in antioxidant defense have been shown to effectively ameliorate bleomycin-induced lung fibrosis in different animal models (3, 20, 137, 215, 245, 246). These data further suggest that ROS/RNS are essential in bleomycin-induced lung fibrosis.

Glutathione and lung fibrosis

Strong evidence that ROS/RNS are involved in the development of lung fibrosis comes from the studies using antioxidants. GSH, a tripeptide, is the most abundant intracellular free thiol and an important antioxidant. GSH participates in diverse biologic processes such as the synthesis of DNA and the metabolism of endogenous and exogenous compounds. However, the most important function of GSH is to detoxify oxidants. GSH concentration in the lung lining fluid has been reported to be 100-fold higher than that in plasma (29), indicating a critical role of GSH in lung antioxidant defense. It has been reported that the GSH concentration is decreased in experimental fibrosis models induced by various stimuli (3, 26, 144, 170) and in the lung-lining fluid in patients with pulmonary fibrotic disorders such as IPF, asbestosis, CF, and ARDS (9–11, 19, 21, 27, 149, 157, 158, 162, 194). The mechanism underlying GSH depletion in fibrotic diseases is unclear. Glutamate cysteine ligase (GCL) is the rate-limiting enzyme in *de novo* GSH synthesis. It has been reported that TGF-β, a most potent profibrogenic cytokine, suppresses the GCL promoter activity and inhibits endogenous GCL mRNA and protein expression, associated with a decline in the intracellular GSH level and an increase in cellular reactive oxygen species in different types of cells (2, 7). Tiitto *et al.* (229) further showed that GCL expression is low in the fibrotic areas of IPF disease. These data suggest that decreased GSH biosynthesis capacity may underlie the decrease in GSH concentrations observed in these diseases.

Although the mechanism and biologic significance of GSH depletion in fibrotic diseases has not been fully elucidated, GSH and *N*-acetylcysteine (NAC), a precursor of GSH, have been used clinically for the treatment of fibrotic diseases (10, 15, 22, 52, 82, 88, 157, 230). It has been reported that aerosol administration of GSH or NAC restored GSH concentration in lung-lining fluid and slowed the deterioration of lung functions in IPF and CF patients (15, 52, 82, 88, 230), indicating a potential therapeutic value of GSH/NAC in the treatment of lung fibrotic diseases. Several studies using bleomycin-induced lung fibrosis models also showed that NAC, when given orally or by aerosol inhalation, attenuated bleomycin-induced lung fibrosis in mice and rats (41, 87, 153, 246). These data further suggest that oxidative stress resulted from increased ROS/RNS production and that decreased GSH concentration contributes importantly to the development of fibrosis.

The mechanism underlying therapeutic/antifibrotic effects of GSH/NAC remains to be further explored. In previous studies, we showed that TGF-β decreased GSH and stimulated ROS production in murine embryo fibroblasts (NIH3T3 cells), whereas exogenous GSH abrogated TGF-β–induced collagen accumulation (138). Furthermore, we demonstrated that exogenous GSH or GSH ester selectively inhibited TGF-β–induced PAI-1 expression and restored tPA and plasmin activities, as well as the collagen degradation rate in TGF-β–treated fibroblasts (235). Most important, we showed that inhibition of plasminogen activation with tranexamic acid or the addition of active PAI-1 to a culture medium almost completely eliminates the restorative effects of GSH on collagen degradation in TGF-β–treated cells. As TGF-β is a most potent and ubiquitous profibrogenic cytokine and its expression is increased in almost all fibrotic diseases, our results suggest that GSH or NAC may exert antifibrotic/therapeutic effects by blocking TGF-β signaling, inhibiting PAI-1 expression, and stimulating collagen degradation (Fig. 3).

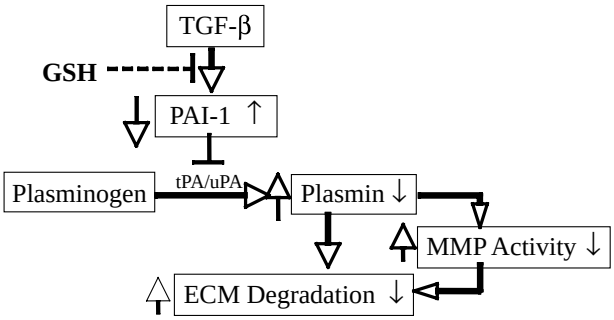

FIG. 3. Potential mechanism whereby GSH inhibits TGF-β–induced collagen accumulation in NIH3T3 cells. GSH inhibits TGF-β–induced PAI-1 expression and therefore increases plasmin formation and collagen degradation.

REDOX REGULATION OF PLASMINOGEN ACTIVATOR INHIBITOR 1 GENE EXPRESSION

Regulation of PAI-1 activity at protein level

Active PAI-1 has a very short half-life, and plasma PAI-1 concentration is low (6–80 ng/ml) under physiologic conditions; however, PAI-1 expression is rapidly induced upon stimulation, suggesting a high synthesis rate and tight control of PAI-1 gene expression. Many types of cells, including endothelial cells, adipocytes, fibroblasts, hepatocytes, smooth muscle cells, and epithelial cells, can synthesize PAI-1. The production of PAI-1 by these and other cells can be induced by various growth factors such as TGF-β and basic fibroblast growth factor, insulin-like growth factor, by cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6), and by hormones such as corticosteroids (51, 54, 57, 141, 142). PAI-1 is synthesized and secreted by cells as an active form; however, two different activity states, active and latent, have been detected *in vivo* (51, 92, 133). The active inhibitory form of PAI-1 spontaneously converts to a latent conformation that can be partially reactivated by denaturing agents (133, 165). In addition to converting to latent PAI-1, which can be reactivated, the active inhibitory form of PAI-1 can be converted to the substrate form, which is irreversibly degraded by its target proteinases (76, 135). The biologic functions of latent and substrate forms of PAI-1 are unclear.

Although factors controlling the conversion of different forms of PAI-1 under physiologic conditions are unknown, it has been reported that oxidants can inactivate PAI-1 activity *in vitro* (131, 218). Lawrence *et al*. (131) showed that the rapidly acting PAI purified from cultured bovine aortic endothelial cells was inactivated by chloramine T under conditions that specifically oxidize methionine and cysteine residues. Both the PAI inhibitory activity and the ability of the PAI to form complexes with tPA were decreased in a dose-dependent manner by chloramine T. Incubation of the chloramine T–inactivated PAI with methionine sulfoxide peptide reductase in the presence of dithiothreitol (DTT) restored >90% of the PAI activity, although little activity was restored by either the reductase or DTT alone, indicating that the oxidation of at least one critical methionine residue is responsible for the loss of PAI activity (131). Strandberg *et al*. (218) showed, however, that mutant proteins of PAI-1, in which Met347 and either of two other methionines, Met266 or Met354, have been replaced with oxidation-resistant valine residues, were equally sensitive to oxidation as wild-type PAI-1, suggesting that a specific oxidation of the methionine residues is not responsible for the inactivation (218). They further showed that when PAI-1 was oxidized, it underwent a rapid conformational change that correlated to the loss of inhibitory activity, suggesting that the increased sensitivity to oxidation may be caused by a conformational change in the inhibitor molecule (218). Nonetheless, to uncover the molecular mechanism whereby PAI-1 activity is regulated under physiologic and pathologic conditions will greatly benefit the development of the therapeutic agents for the treatment of fibrotic diseases in which PAI-1 plays a pivotal role.

Redox regulation of PAI-1 gene expression

Although the PAI-1 activity can be modified at the protein level, the regulation of PAI-1 is achieved mainly by alteration in the rate of PAI-1 gene expression (103). The human PAI-1 gene is ~12.2 kb in length, composed of nine exons and eight introns. A similar structure has been found with rat and mouse PAI-1 genes (23, 190). The 5′-flanking region of the human PAI-1 gene contains a perfect "TATA" box and several transcription factor binding sites, including binding sites for Smads, activator protein 1 (AP-1), SP-1, hypoxia-induced factor–responsible element (HREs), and NF-κB (38, 97, 115, 164, 237). The transcription factor binding sites identified in the rat PAI-1 promoter show a high degree of sequence similarity to sequences in the mouse (190) and human (23, 36, 110) PAI-1 promoter, suggesting that they are regulated by similar mechanisms. PAI-1 is involved in many physiologic and pathologic processes, and its gene expression is induced by numerous stimuli. In this section, we focus only on the redox regulation of PAI-1 gene expression by several important growth factors/cytokines/agents that are involved in the development of fibrosis.

Transforming growth factor beta (TGF-β). Although various cytokines, chemokines, and growth factors have been shown to play important roles in the development of fibrosis, TGF-β is considered to be the most potent and ubiquitous profibrogenic cytokine. TGF-β induces PAI-1 gene expression in various types of cells (37, 44, 85, 94, 125, 204, 244); an elevated PAI-1 level is also associated with increased TGF-β expression and ECM deposition under diverse pathologic conditions (48, 65, 79, 90, 145, 209, 232), indicating a critical role of PAI-1 in TGF-β fibrogenesis. TGF-β induces its target genes mainly through the Smad signaling pathway. Induction of PAI-1 by TGF-β through the Smad pathway has been well described in the past decade. On the binding of TGF-β to the membrane receptor, TGF-β signaling is transduced to the nucleus by a series of events involving phosphorylation of Smad 2/3 and nuclear translocation of Smad 2/3 and Smad 4 complex. Binding of the Smad2/3 and Smad 4 complex to Smad-binding sites in the promoter of the PAI-1 gene initiates the gene transcription.

Importantly, it has been reported that TGF-β increases ROS production (74, 93, 96, 105, 111, 169, 227), decreases GSH concentration (2, 7, 71, 107, 205), and suppresses the activities of antioxidant enzymes such as catalase, superoxide dismutase (SOD), and glutathione peroxidase (105, 114) in various types of cells and *in vivo*. Many of the effects of TGF-β (68, 74, 109, 111, 132, 205, 223, 255), including induction of PAI-1 (109, 207), are indeed mediated by ROS, although the underlying molecular mechanism is still not completely understood. In addition to the Smad pathway, several other pathways, including the mitogen-activated protein kinase (MAPK) pathway, also play a role in TGF-β induction of PAI-1 expression in various types of cells (73, 85, 94, 125, 204, 241, 244). As MAPK pathways are sensitive to the redox state of cells, it is hypothesized that ROS/RNS may mediate TGF-β induction of PAI-1 expression through activating MAPKs. Nevertheless, although it has been well documented that TGF-β increases ROS production, activates MAPK pathways, and induces PAI-1, whether ROS mediate the induction of PAI-1 by TGF-β through activating MAPKs and which component(s) in MAPK pathways is (are) the redox sensor, however, are unclear.

In an effort to elucidate the potential link between ROS production, MAPK activation, and the PAI-1 induction by TGF-β, we systematically studied the effects of the specific inhibitors and dominant negative mutants for JNK, p38, ERK MAPKs on TGF-β–induced PAI-1 expression, as well as the effecs of ROS scavengers, including GSH on TGF-β–induced MAPK activation and PAI-1 expression in NIH3T3 cells. Our results show that TGF-β increases ROS production, activates JNK and p38 pathways, but not ERK pathway, in NIH3T3 cells, associated with an induction of PAI-1 expression (234). JNK/p38 pathway inhibitor or dominant negative mutant significantly reduces TGF-β–induced PAI-1 expression at both mRNA and protein levels (234). Most importantly, inhibition of NADPH oxidase, which is responsible for the production of ROS in TGF-β–treated cells, or treatment of the cells with exogenous GSH, almost completely blocks TGF-β–induced JNK and p38 phosphorylation and partially inhibits TGF-β–induced PAI-1 expression (234). These results support the notion that MAPK pathways are involved in the induction of PAI-1 by TGF-β through a redox-sensitive mechanism.

The human PAI-1 promoter region close to the TATA box contains two TRE-like elements. The c-Jun homodimers and c-Jun/c-Fos heterodimers bind to these elements to mediate TGF-β responses (115). It has been reported that the transcriptional activation by Smad is mediated through the AP-1 transcription factor complex (85, 247). It has also been shown that ATF-2, a downstream substrate of both JNK and p38, participates in transcription complexes in association with Smad proteins (206). Two SP-1 binding site–like sequences are also identified at -72 to -67 and -45 to -40 in the proximal promoter region of the PAI-1 gene (38). It has been reported that TGF-β upregulates PAI-1 gene expression by activating SP-1–dependent transcription through the induction of Smad/SP-1 complex formation (44, 98). These data suggest that oxidants may mediate TGF-β–induced PAI-1 expression by activating MAPK pathways, which then facilitate the binding of Smad proteins to the PAI-1 promoter and activate PAI-1 gene expression. This notion is supported by our recent findings that, although GSH has no effect on TGF-β–induced Smad 2/3 phosphorylation or pSmad 2/3 and Smad 4 nuclear translocation, it blocks the binding of the transcription factors to not only the AP-1 and SP-1 but also Smad *cis*-elements in the PAI-1 promoter (234). Blocking the transcription factors AP-1, SP-1, and Smad ODNs with decoy oligonucleotides, on the other hand, abrogates the inhibitory effect of GSH on TGF-β–induced PAI-1 promoter activity (234).

Tumor necrosis factor alpha (TNF-α). TNF-α is another important growth factor involved in the development of fibrosis under many pathologic conditions. The expression of TNF-α is increased in various lung fibrotic diseases (18, 179,

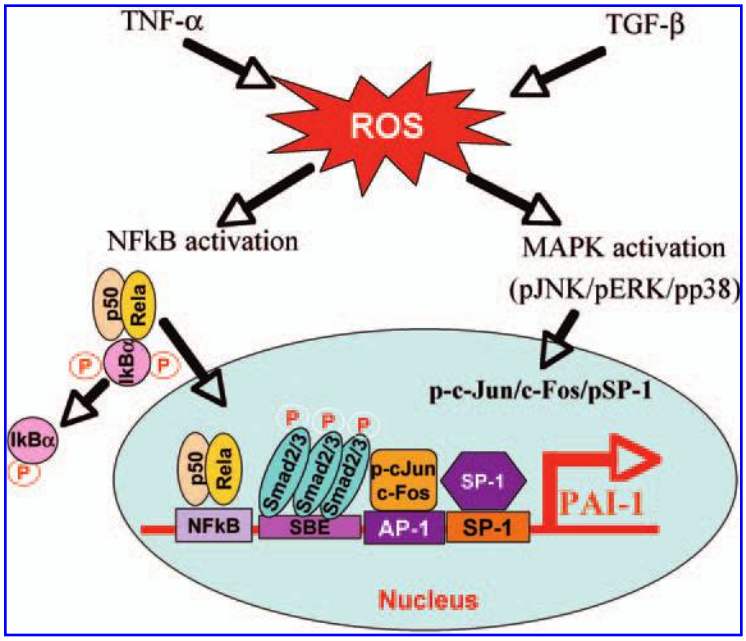

FIG. 4. Potential signaling pathways mediating ROS induction of PAI-1 by TGF-β and TNF-α. TGF-β and TNF-α increase ROS production, which, by activating the MAPK pathways and/or the NF-κB pathway, increase the transcription rate of the PAI-1 gene.

250, 252), and importantly, the elevation of TNF-α level in many fibrotic diseases is companied by an increase in PAI-1 expression (202, 225, 242). *In vitro* studies further show that TNF-α induces PAI-1 in various types of cells (37, 97, 146, 180, 203, 223, 224, 226) and ROS mediate such induction (174, 203, 223). Swiatkowska *et al*. (223) reported that PAI-1 expression in endothelial cells is upregulated by TNF-α or H_2O_2, which was reversed when NAC was added to the culture medium. NAC also blocked the PAI-1 promoter activity stimulated by TNF-α (223). Although it is not clear which signaling pathway(s) mediates TNF-α induction of PAI-1 expression through a redox-sensitive mechanism, several studies have suggested that NF-κB may be involved. Swiatkowska *et al*. (224) showed that TNF-α induced PAI-1 gene expression through a regulatory region present in segment $-664/-680$ of the PAI-1 promoter, which interacts with NF-κB, and that ROS mediate such induction (224). Hou *et al*. (97) also showed that a TNF-α–responsive enhancer located 15 kb upstream of the transcription start site in the PAI-1 gene, which contains a conserved NF-κB-binding site, mediated TNF-α response in bovine aortic endothelial cells. Conversely, Takeshita *et al*. (226) showed that protein kinase C, MAPK, protein tyrosine kinase, and NF-κB pathways are all involved in the induction of PAI-1 in TNF-α–treated nonmalignant human hepatocyte cells. As the NF-κB pathway is sensitive to ROS, these data suggest that ROS/RNS mediate the induction of PAI-1 by TNF-α, probably by altering the NF-κB pathway signaling (Fig. 4).

Angiotensin II (Ang II). Ang II has numerous biologic functions and is involved in many pathologic conditions, including the development of fibrosis (104, 108, 112, 113, 119, 211, 221). It has been reported that Ang II induces ECM accumulation by a mechanism independent of its vasopressor effect (156), probably by stimulating PAI-1 expression and therefore inhibiting ECM degradation (108, 112, 113, 119, 173, 238). Oikawa *et al*. (173) reported that angiotensin-converting enzyme inhibitors (ACEIs; captopril or enalapril) or angiotensin II receptor antagonist (AIIRA, L158,809) markedly attenuated increased PAI-1 mRNA expression and reduced glomerular lesions (thrombosis, mesangiolysis, and sclerosis index) in a radiation-induced kidney fibrosis model. The data suggest that inhibition of the renin–angiotensin system may ameliorate radiation-induced injury by suppressing PAI-1 expression and thus accelerating ECM degradation. Jesmin *et al*. (108) further reported that the expression of PAI-1 in coronary vessels and the perivascular area was increased in diabetic hearts, which was associated with reduced activities of MMP-2 and membrane type-1 MMP (MT1-MMP) and increased deposition of collagen type I and III as well as fibrin. Such an increase in PAI-1 expression and ECM deposition was reversed to nondiabetic levels by the angiotensin II type 1 receptor blocker candesartan (108). These results suggest that increased production of Ang II causes coronary matrix remodeling in insulin-resistant diabetes at least in part by increasing PAI-1 expression and thus decreasing MMP-2 and MT1-MMP activities (108). Although the molecular mechanism whereby Ang II induces PAI-1 expression has not been completely elucidated, several studies have shown that Ang II increases ROS/RNS production, which mediates the induction of PAI-1 by Ang II in different types of cells (124, 248, 249). It has been reported that Ang II increased ROS production in adipose tissue/adipocytes (124) and in rat aorta endothelial cells (248, 249), whereas olmasartan, an angiotensin II (Ang II) type-1 receptor blocker (124), and NAC (248, 249) suppressed Ang II–stimulated ROS production and PAI-1 expression.

Radiation. Although the underlying mechanism is still not well understood, it has been proposed that free radicals produced by radiation contribute importantly to radiation-associated fibrosis, in part by inducing PAI-1 expression. Zhao *et al*. (254, 255) showed that irradiating rat kidney tubule epithelial cells (NRK52E) with 1 to 20 Gy gamma-rays led to dose-dependent increases in steady-state levels of PAI-1 mRNA and protein within 24 and 48 h, respectively, whereas enhancement of intracellular soluble thiol pools with NAC abolished the radiation-induced upregulation of PAI-1. Overexpression of catalase also inhibited radiation-induced PAI-1 expression, suggesting a mechanistic role for hydrogen peroxide in the induction of PAI-1 expression during radiation exposure (255). Hageman *et al*. (86) demonstrated that irradiation of HepG2 cells leads to a significant increase in PAI-1 mRNA levels, whereas TGF-β shows strong cooperative effects with radiation in activating the PAI-1 gene (86). Schultze-Mosgau *et al*. (208) further showed that radiation induced PAI-1 expression in the skin of Wistar rats by increasing TGF-β expression as anti-TGF-β antibody blocked radiation-induced PAI-1 expression and skin fibrosis. Damage to the skin ECM is the hallmark of long-term exposure to solar UV radiation. Seite *et al*. (210) showed that a single minimal erythemal dose of UV significantly enhanced IL-1α, IL-1β, and PAI-1 mRNA levels in human skin *in vivo* ($p < 0.05$), indicating that increased PAI-1 expression is involved in UV-light–induced skin pathology. Nevertheless, as in many other situations, the signaling pathway(s) mediating ROS induction of PAI-1 expression in radiation-treated cells or animals remain largely unknown.

CONCLUSIONS

PAI-1 plays a pivotal role in the development of lung fibrosis. Various growth factors, cytokines, and chemical/physical agents can induce PAI-1 expression, and ROS/RNS mediate the induction of PAI-1 by many of these stimuli. Although MAPK and NF-κB pathways have been shown to be redox sensitive and may be the downstream effectors of ROS/RNS in the induction of PAI-1 by different stimuli, the molecular mechanism underlying PAI-1 induction by ROS/RNS remains largely unknown. As PAI-1 plays a critical role in the development of fibrosis, not only in the lung but also in many other organs, and increasing ROS/RNS production is a common feature for many fibrogenic agents, to uncover the mechanism underlying the induction of PAI-1 by ROS/RNS will provide important information not only for understanding the pathogenesis of these fibrotic diseases but also for the development of effective therapeutic agents for the treatment of these devastating diseases.

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ABBREVIATIONS

ACE, angiotensin-converting enzyme; AP-1, activator protein 1; Ang II, angiotensin II; ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; CF, cystic fibrosis; DTT, dithiothreitol; ECM, extracellular matrix; ELF, epithelial lining fluid; ERK, extracellular signal regulated kinase; GSH, glutathione; HGF, hepatocyte growth factor; IPF, idiopathic pulmonary fibrosis; JNK, c-Jun NH_2-terninal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NAC, *N*-acetylcysteine; NF-κB, nuclear factor κB; PA, plasminogen activator; PAI-1, plasminogen activator inhibitor 1; ROS/RNS, reactive oxygen/nitrogen species; SBE, Smad binding element; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

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