Bleomycin-induced Pulmonary Fibrosis in Transgenic Mice That either Lack or Overexpress the Murine Plasminogen Activator Inhibitor-1 Gene

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Abstract

Impaired fibrinolytic activity within the lung is a common manifestation of acute and chronic inflammatory lung diseases. Because the fibrinolytic system is active during repair processes that restore injured tissues to normal, reduced fibrinolytic activity may contribute to the subsequent development of pulmonary fibrosis. To examine the relationship between the fibrinolytic system and pulmonary fibrosis, lung inflammation was induced by bleomycin in transgenic mice that either overexpressed or were completely deficient in murine plasminogen activator inhibitor-1 (PAI-1). 2 wk after 0.075 U of bleomycin, the lungs of transgenic mice overexpressing PAI-1 contained significantly more hydroxyproline (118±8 μg) than littermate controls (70.5±8 μg, $P < 0.005$). 3 wk after administration of a higher dose of bleomycin (0.15 U), the lung hydroxyproline content of mice completely deficient in PAI-1 (49±8 μg) was not significantly different ($P = 0.63$) than that of control animals receiving saline (37±1 μg), while hydroxyproline content was significantly increased in heterozygote (77±12 μg, $P = 0.06$) and wild-type (124±19 μg, $P < 0.001$) littermates. These data demonstrate a direct correlation between the genetically determined level of PAI-1 expression and the extent of collagen accumulation that follows inflammatory lung injury. These results strongly support the hypothesis that alterations in fibrinolytic activity influence the extent of pulmonary fibrosis that occurs after inflammatory injury. (*J. Clin. Invest.* 1996. 97:232–237.) Key words: fibrinolysis • fibrin • plasmin • interstitial lung disease • collagen

Introduction

Successful repair of damaged alveoli during inflammatory lung diseases requires replacement of injured alveolar cells, restoration of damaged extracellular matrix, and clearance of plasma proteins that have leaked into the alveolar space. Of critical importance to these repair processes is plasmin, a broad spectrum protease which is involved in cell migration, modulation of inflammatory activity, and breakdown of fibrin and other extracellular proteins. This latter function of plasmin may be important for limiting scar formation by dismantling the provisional matrix on which fibroblasts invade and secrete interstitial collagens. The normal alveolar space has net fibrinolytic activity due to the presence of urokinase-type plasminogen activator (u-PA)[1] (1, 2). However, during many acute and chronic inflammatory lung disorders, fibrin accumulates in lung tissue (3–9). The fibrinolytic activity in bronchoalveolar lavage (BAL) fluid from patients with the adult respiratory distress syndrome (1, 2), idiopathic pulmonary fibrosis (10), sarcoidosis (10, 11), and bronchopulmonary dysplasia (12) has been found to be suppressed. All of the above diseases have been associated with the development of pulmonary fibrosis. A similar pattern of depressed fibrinolysis can be seen in a variety of animal models of lung injury (13–15).

Plasminogen activator inhibitor-1 (PAI-1) is the major inhibitor of plasminogen activators in plasma (16) and the alveolar space (2). PAI-1, a member of the serine protease inhibitor (serpin) gene family, rapidly inhibits both u-PA and tissue-type plasminogen activator (t-PA) by forming 1:1 protease–inhibitor complexes that are enzymatically inactive (16). Complete deficiency of PAI-1 in a human was associated with abnormal bleeding (17), and mice that are deficient in PAI-1 display enhanced fibrinolytic activity (18). Conversely, increased plasma PAI-1 levels in humans are associated with thrombotic events (19, 20). Elevated levels of PAI-1 have been observed in BAL specimens obtained from patients with adult respiratory distress syndrome and have been shown to reduce the fibrinolytic capacity of the fluid (1, 2).

Although previous experiments have demonstrated an association of increased PAI-1 levels and pulmonary fibrosis, the primary role of PAI-1 in this process has not been established. To determine whether alterations in systemic PAI-1 levels affect the development of pulmonary fibrosis, lung injury was induced by bleomycin in transgenic mice that were engineered to either overexpress or be completely deficient in PAI-1. The level of PAI-1 gene expression was observed to strongly correlate with the amount of collagen accumulation within lung tissues, suggesting that the balance of fibrinolytic activity within

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1. *Abbreviations used in this paper:* BAL, bronchoalveolar lavage; PAI-1, plasminogen activator inhibitor-1; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

the lung is an important determinant of the pulmonary response to inflammatory injury.

Methods

Animals. All animal experiments were performed in accordance with institutional guidelines set forth by the University committee on the use and care of animals. PAI-1–deficient mice generated by homologous recombination were a generous gift from D. Collen and P. Carmeliet (University of Leuven, Belgium) (21). PAI-1–deficient (−/−) mice, along with PAI-1 heterozygote (+/−) and PAI-1 wild-type (+/+) littermates, were generated by a cross between heterozygote animals. Genotyping was performed by PCR analysis of tail DNA specimens obtained at 3 wk of age (22). Primers which annealed to bp 1007–1031 (5′) and 1503–1532 (3′) of the neomycin resistance gene were used to recognize the knock-out construct, while primers designed to anneal to bp 1263–1286 (5′) and 1473–1496 (3′) (across intron H) of the PAI-1 cDNA sequence were used to recognize the native gene. Thus, DNA samples from mice heterozygous for PAI-1 displayed two different PCR products (496 and 520 bp).

Transgenic mice overexpressing a murine PAI-1 minigene under the direction of the CMV promoter were generated previously (Shen, T., and D. Ginsburg, manuscript in preparation). Serum levels of PAI-1 in mice carrying the PAI-1 transgene were 264±53 vs. 11±4 ng/ml in littermate controls. PAI-1 levels in lung homogenates of transgenic mice were 1,448±320 ng/gram wet tissue compared with 376±38 ng/gram in littermate controls. The PAI-1 transgenic and normal littermate control animals that were used in the experiments were the products of crosses between the transgenic founder animal (SJL/J) and a C57BL/6 mate. The presence of the PAI-1 transgene in individual mice was determined using PCR with the PAI-1 primers as described above. Two products of different sizes were generated in the presence of transgene: a 496-bp product from the normal allele, and a 233-bp product from the transgene, which lacks an intervening intron. Only the 496-bp product was observed in animals lacking the transgene.

Bleomycin treatment. Male and female 4–6-wk-old mice, weighing an average of 20±3 grams, were maintained in a specific pathogen-free environment and fed ad libitum. Intratracheal administration of bleomycin to mice under intraperitoneal ketamine anesthesia was performed with the operator blinded to genetic status of mice, as described previously (23). After tracheostomy, bleomycin (Bristol Laboratories, Syracuse, NY) was instilled at doses of 0.075 or 0.15 U in a final volume of 50 μl. These doses were determined from preliminary experiments with mice of similar genetic background to consistently produce pulmonary fibrosis with a low mortality rate (< 25%).

Hydroxyproline assay. To estimate total lung collagen content, hydroxyproline was measured as previously described with modifications (24). The lung vasculature was perfused free of blood by slowly injecting 3 ml of PBS into the right ventricle. The left lung was then excised and homogenized (Tissue Tearor; Biospec Products, Inc., Bartlesville, OK) in 2 ml of PBS. A 1-ml aliquot was desiccated using rotary vacuum pump (Savant Instruments, Inc., Farmingdale, NY) and then hydrolyzed in 6 N HCl at 110°C for 12 h. 50-μl aliquots were added to 1 ml of 1.4% chloramine T (Sigma Chemical Co., St. Louis, MO), 10% *n*-propanol, and 0.5 M sodium acetate, pH 6.0. After 20 min of incubation at room temperature, 1 ml of Erlich's solution (1 M *p*-dimethylaminobenzaldehyde in 70% *n*-propanol, 20% perchloric acid) was added and allowed to incubate at 65°C for 15 min. Absorbance was measured at 550 nm and the amount of hydroxyproline was determined against a standard curve generated using known concentrations of reagent hydroxyproline (Sigma Chemical Co.). The ability of the assay to completely hydrolyze and recover hydroxyproline from collagen was confirmed using samples containing known amounts of purified collagen (Vitrogen-100; Celtrix Laboratories, Palo Alto, CA).

Histology and immunohistochemistry. After the left lung of each animal was removed for hydroxyproline assay, 0.5 ml 4% paraformaldehyde was instilled intrabronchially into the right lung, the airway was ligated, and the lung was submerged in the same fixative. The tissue was embedded in paraffin, and histologic sections were viewed after Masson trichrome staining. Immunostaining of fibrin was performed on paraffin-embedded sections that had been fixed in 4% paraformaldehyde. Goat anti–mouse serum was purchased from Nordic Immunological Laboratories (Capistrano Beach, CA). The IgG fraction was purified by passage over a protein A–Sepharose column (Pharmacia, Uppsala, Sweden) and used at a concentration of 10 μg/ml. Binding of primary antibody was detected using a biotinylated rabbit anti–goat antibody and a Histostain-SP Kit (Zymed Laboratories, South San Francisco, CA). To evaluate the specificity of the immunostaining, preimmune goat IgG (10 μg/ml) was used as a control condition.

Statistics. Values are expressed as mean±SEM. Differences between genetically altered mice and littermate controls were analyzed using ANOVA with Fisher's PLSD test for pair-wise comparisons (Statview 4.0; Abacus Concepts, Inc., Berkeley, CA). A P value < 0.05 was considered statistically significant.

Results

Collagen content of bleomycin-treated lungs. 2 wk after intratracheal bleomycin (0.075 U), lung collagen, as measured by hydroxyproline content, increased in both PAI-1 overexpressing mice ($P < 0.001$) and their normal littermate controls ($P < 0.03$) (Fig. 1). However, the increase in collagen induced by

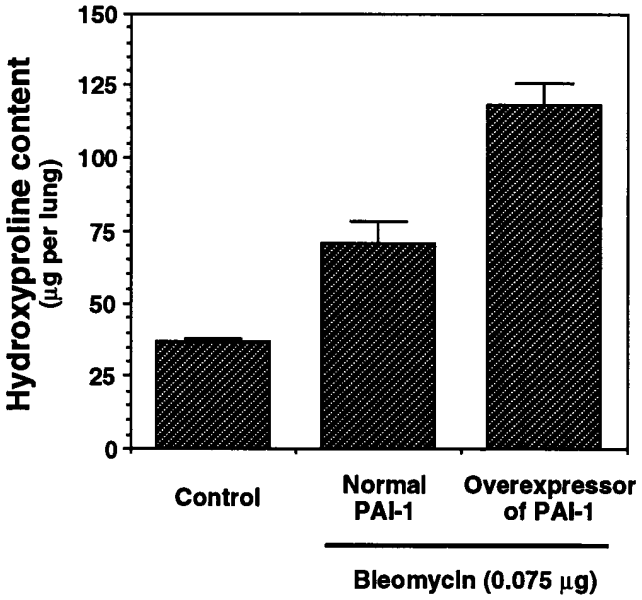

Figure 1. Effect of intratracheal bleomycin on lung hydroxyproline content in PAI-1–overexpressing mice and normal littermates. Bleomycin (0.075 U) or PBS was instilled intratracheally into normal or transgene PAI-1–overexpressing mice, and 2 wk later lung hydroxyproline content was measured as outlined in Methods. The bleomycin-induced increase in lung hydroxyproline of transgenic mice overexpressing PAI-1 ($n = 3$) was greater ($P < 0.002$) than that seen in the bleomycin-treated normal PAI-1 littermates ($n = 3$). The hydroxyproline content of both bleomycin-treated groups was greater ($P < 0.005$) than that of PBS-treated control mice lacking the overexpressing murine PAI-1 transgene ($n = 2$). Data from other experiments revealed that lung hydroxyproline content did not differ between normal and PAI-1–overexpressing mice treated with PBS.

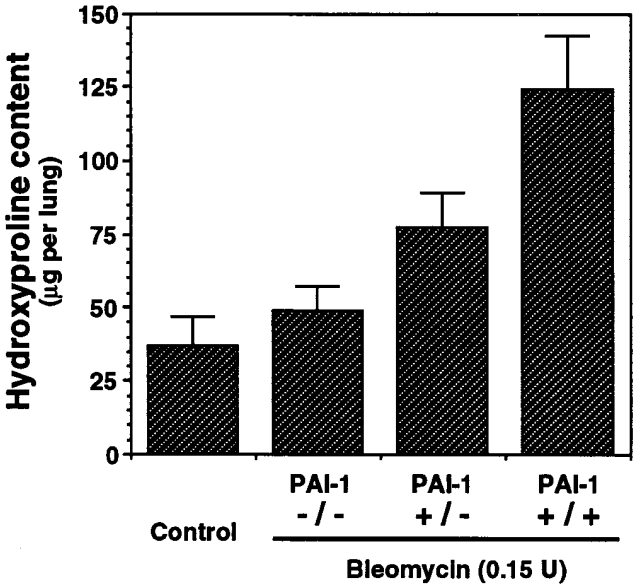

Figure 2. Effect of intratracheal bleomycin on lung hydroxyproline content in PAI-1–deficient mice and littermates. Bleomycin (0.15 U) or PBS was instilled intratracheally into normal (PAI-1 +/+), heterozygous PAI-1–deficient (PAI-1 +/−), or homozygous PAI-1–deficient (PAI-1 −/−) mice. 3 wk later, lung hydroxyproline content was measured as outlined in Methods. The lung hydroxyproline content of bleomycin-treated mice was directly related to PAI-1 gene dose ($r = 0.72$, $P < 0.001$) with the level in normal (PAI-1 +/+) mice being significantly greater than PAI-1 +/− ($n = 6$, $P < 0.02$) and PAI-1 −/− ($n = 6$, $P < 0.001$) mice. The hydroxyproline content of bleomycin-treated PAI-1 −/− mice was not significantly different from that of PBS-treated PAI-1 +/− littermates (*Control*, $n = 4$, $P < 0.63$).

bleomycin in mice carrying the PAI-1 transgene (3.2 times PBS control animals) was significantly greater ($P < 0.005$) than the increase seen in the transgene-negative littermates (1.9 times PBS controls). No significant differences in hydroxyproline content were observed between normal and transgene-positive mice when no treatment or only PBS was administered (data not shown).

Mice deficient in PAI-1 were next studied to determine if they would develop less collagen in response to bleomycin. For these experiments a higher dose of bleomycin (0.15 U) was used to more clearly discern a protective effect of PAI-1 deficiency. 3 wk after wild-type (PAI-1 +/+) mice were given bleomycin, lung hydroxyproline content increased significantly compared with mice that received only PBS ($P < 0.001$) (Fig. 2). In contrast, PAI-1 −/− mice appeared to be largely protected from the fibrotic effects of bleomycin. The lung hydroxyproline content of bleomycin-treated PAI-1 −/− mice was markedly lower than that of bleomycin-treated PAI-1 +/+ mice ($P < 0.001$) and was not significantly different from the level of mice receiving only PBS ($P = 0.63$). The hydroxyproline content observed in PAI-1 +/− mice treated with bleomycin was intermediate, revealing a direct correlation between PAI-1 gene dose and lung hydroxyproline content ($r = 0.72$; $P = 0.001$). No difference in hydroxyproline content of PAI-1 −/− and PAI-1 +/+ mice was observed in the absence of bleomycin treatment (data not shown).

Histology. To provide a visual correlate to the quantitative hydroxyproline data, Masson trichrome–stained sections of lung tissue were examined using light microscopy. The lungs of animals receiving intratracheal PBS appeared normal, regardless of their PAI-1 status. Stained collagen in the lungs of these animals was detectable only in thin bands immediately adjacent to large vessels and airways (data not shown). After 0.075 U bleomycin, the lungs of transgenic mice overexpressing PAI-1 contained dense bands of collagen replacing large areas of lung parenchyma, an example of which is shown in Fig. 3 *B*. The areas of abnormal stainable collagen in the lungs of normal mice receiving the same dose of bleomycin were fewer in number and considerably less dense (Fig. 3 *A*). When a higher dose of bleomycin (0.15 U) was administered to normal PAI-1 +/+ mice, a severe fibrotic response was seen. The lungs of these animals contained multiple areas of darkly stained collagen as demonstrated in Fig. 3 *C*. In marked contrast, the lung tissue of PAI-1 −/− mice receiving 0.15 U of bleomycin appeared essentially normal (Fig. 3 *D*). Of note, areas of lung tissue with increased collagen also contained increased numbers of inflammatory cells.

Immunohistochemical staining for fibrin. To determine if fibrin accumulation in lung parenchyma correlated with the extent of fibrosis and PAI-1 gene status, lung sections were stained with an antifibrin antibody (Fig. 4). Immunostainable fibrin that was contained in lungs of PAI-1–overexpressing, bleomycin-treated (0.075 U) mice appeared much more abundant than that of bleomycin-treated, normal littermates. The red-staining fibrin was present in both airspaces and interstitium and was located within and adjacent to areas of inflammation and fibrosis. Wild-type mice (PAI-1 +/+) receiving a higher dose of bleomycin (0.150 U) also showed extensive areas of fibrin deposition, while PAI-1 −/− littermates receiving the same dose of bleomycin had no fibrin staining. Mice that received only PBS had no stainable fibrin. Serial sections from each of the tissue blocks had no red-staining when exposed to preimmune serum.

Discussion

The normal alveolar space has net fibrinolytic activity and is proficient at clearing extravascular fibrin by producing u-PA (1, 2). However, during both acute and chronic inflammatory lung diseases, the fibrinolytic process becomes impaired due to increased levels of inhibitors to plasminogen activators (e.g., PAI-1) and plasmin. Plasmin and/or plasminogen activators can participate in many processes including cell migration (25), extracellular matrix remodeling (26–30), generation of inflammatory peptides (31, 32), and activation of latent cytokines and enzymes including metalloproteinases such as collagenases (26, 29). Therefore, we hypothesized that the degree of pulmonary fibrosis that follows inflammatory lung injury would be influenced by the state of the fibrinolytic system. To test this hypothesis, we used transgenic mice with altered PAI-1 gene expression to examine the relationship between the fibrinolytic system and the development of pulmonary fibrosis after inflammatory injury.

PAI-1, a rapid inhibitor of u-PA and t-PA activity, occurs in the lung either as a product of pulmonary alveolar epithelial cells (33, 34) or by transfer from plasma where it is a normal constituent. Transgenic animals that are either deficient in or overexpress PAI-1 have been used to study the functional role of PAI-1. Mice totally deficient in PAI-1 appear normal under basal conditions (18). However, when preformed clots are embolized to their lungs, the clots are lysed more rapidly than

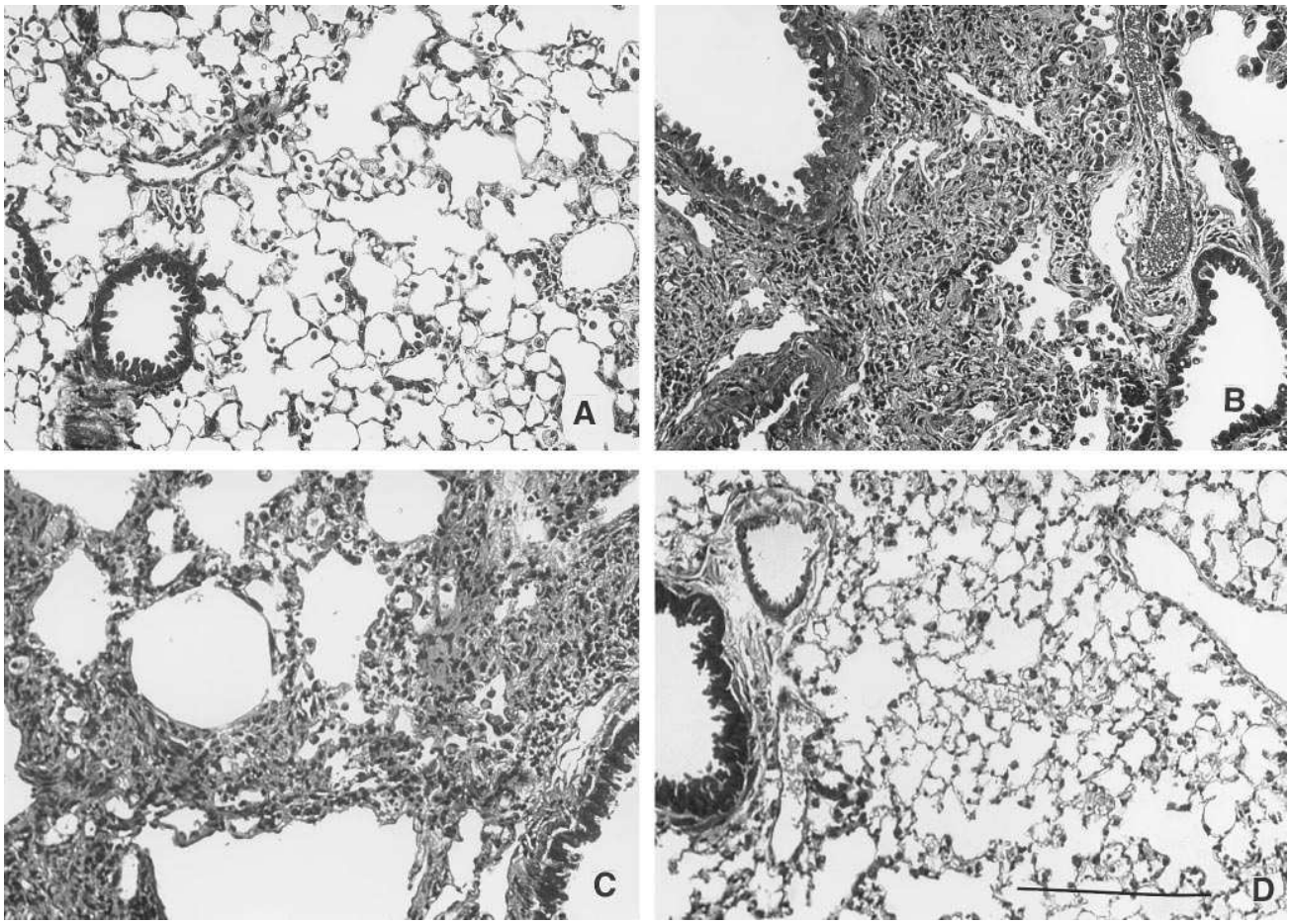

Figure 3. Photomicrographs of Masson trichrome–stained sections of lung tissue from bleomycin-treated mice. (*A*) Normal mice 2 wk after 0.075 U bleomycin. (*B*) PAI-1–overexpressing mice 2 wk after 0.075 U bleomycin. (*C*) Normal mice (PAI-1 +/+) 3 wk after 0.15 U bleomycin. (*D*) PAI-1 homozygous deficient mice (PAI-1 −/−) 3 wk after receiving 0.15 U bleomycin. The photomicrographs were selected to illustrate the pattern and extent of fibrosis present in the abnormal regions of lung for each experimental group. All panels are at the same magnification; bar, 200 μm.

they are in control animals. In addition, the PAI-1–deficient animals are less likely to develop venous thrombi after foot pad injection of endotoxin. Conversely, mice expressing high levels of a human PAI-1 transgene develop spontaneous thromboses within their tail and hind leg veins (35). Since these studies showed that fibrin clearance can be influenced by manipulations in PAI-1 gene expression, this approach appeared suitable for investigating the linkage between fibrinolysis and the development of pulmonary fibrosis. The importance of the plasminogen activation system in clearance of fibrin from the lung is further demonstrated by the observation that mice with combined deficiency of t-PA and u-PA, the two major plasminogen activators, exhibit spontaneous fibrin deposition in lung tissue (36).

Bleomycin administration to animals has been widely used to study the pathogenesis of pulmonary fibrosis (14, 23, 37–39). When instilled intratracheally into mice, bleomycin causes a pneumonitis that progresses to fibrosis in a dose-dependent manner with increased collagen content occurring as early as 2 wk after instillation (23). The fibrogenic effects of bleomycin can be attenuated by suppressing immune or inflammatory responses (23, 40, 41). Lung tissue obtained from bleomycin-injured animals has histologic features that are similar to those seen in the lungs of humans with the adult respiratory distress syndrome and idiopathic pulmonary fibrosis. Of particular relevance to the present study, bleomycin treatment of animals suppresses the fibrinolytic activity of BAL fluid in a pattern similar to that seen in human inflammatory lung diseases (14, 38). Furthermore, intratracheal administration of recombinant human u-PA to rats 30 d after bleomycin exposure can temporarily reduce the amount of lung fibrosis (39).

The results of this study demonstrate a strong relationship between PAI-1 gene dose and the degree of pulmonary fibrosis that follows bleomycin administration. In particular, PAI-1–overexpressing mice experienced greater fibrosis than wild-type mice, while mice homozygous deficient for PAI-1 were protected from fibrosis, with heterozygotes showing an intermediate effect. As mentioned above, there are multiple mechanisms by which alterations in fibrinolytic activity could influence the development of fibrosis. One such mechanism is the ability of plasmin to degrade extravascular fibrin and thus remove the provisional matrix on which fibroblasts invade to form collagen. In support of this, we found that fibrin deposits were localized within and around areas of fibrotic lung. However, other mechanisms might link the fibrinolytic system to scar formation. Since the intensity of bleomycin-induced in-

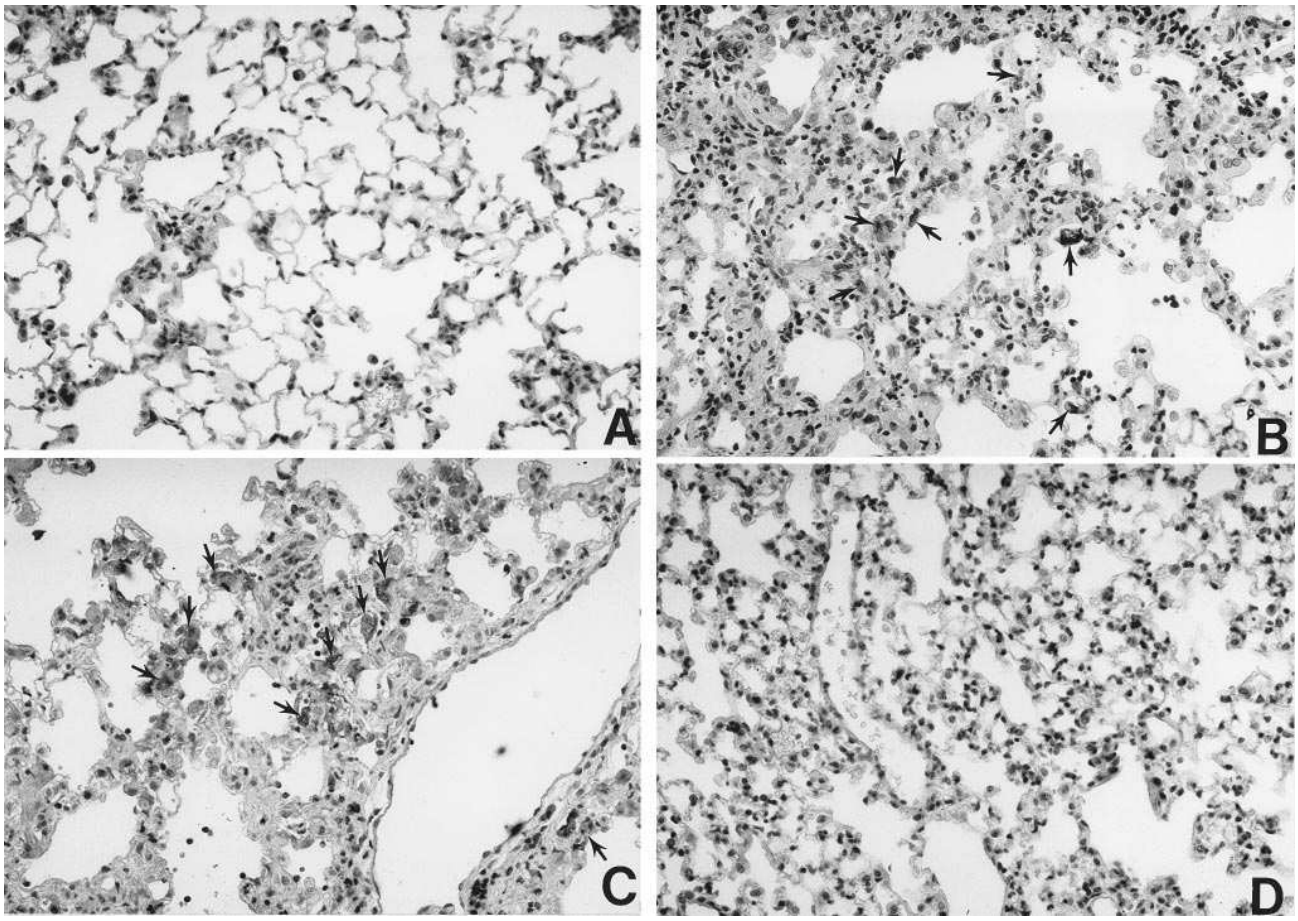

Figure 4. Photomicrographs of immunostained lung tissue from bleomycin-treated mice localizing sites of fibrin deposition. (*A*) Normal mice 2 wk after 0.075 U bleomycin. (*B*) PAI-1–overexpressing mice 2 wk after 0.075 U bleomycin. (*C*) Normal mice (PAI-1 +/+) 3 wk after 0.15 U bleomycin. (*D*) PAI-1 homozygous deficient mice (PAI-1 −/−) 3 wk after receiving 0.15 U bleomycin. Arrows indicate material staining positively with a goat antiserum against murine fibrin. No antibody binding was seen using control antiserum. The photomicrographs were selected to illustrate the pattern and extent of fibrin deposition in the abnormal regions of lung for each experimental group. All panels are at the same magnification; bar, 200 μm.

flammation is known to correlate with the degree of subsequent fibrosis (42), the fibrinolytic system may exert its effect in part by modulating various inflammatory processes mentioned above. Although plasmin activation has been implicated in the process of inflammatory cell migration, overexpression of PAI-1 does not appear to prevent accumulation of leukocytes in bleomycin-treated mice. Apparently, residual or redundant proteolytic mechanisms are adequate to allow inflammatory cells to infiltrate the lungs.

In summary, we have shown that alterations in the fibrinolytic environment of the alveolar space during inflammatory injury influence the subsequent development of pulmonary fibrosis. These findings suggest that therapeutic interventions designed to enhance fibrinolysis such as administration of either plasminogen activators (39) or inhibitors of PAI-1 (43) may limit the development of pulmonary fibrosis that occurs as a consequence of acute or chronic inflammatory lung diseases.

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