Reduction in Fibrotic Tissue Formation in Mice Genetically Deficient in Plasminogen Activator Inhibitor-1

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Mice with homozygous deletion of the plasminogen activator inhibitor-1 gene (PAI-1^{-/-}) are relatively protected from bleomycin-induced pulmonary fibrosis. At least part of the protective effect appears to occur during the latter stages of the pathological process when fibrotic tissue is being deposited. To investigate the effect of PAI-1 deficiency on fibrosis, we studied the accumulation of fibrotic tissue within subcutaneously implanted polyvinyl alcohol sponges. Similar to the effect of PAI-1 deficiency on bleomycin-induced pulmonary fibrosis, the accumulation of fibrotic tissue within implanted sponges occurred more slowly in PAI-1^{-/-} compared to wild-type mice. Another striking difference observed in the PAI-1^{-/-} mice was the rapid removal of a fibrin-rich matrix that formed within the sponges by 1 day after implantation in both wild-type and PAI-1^{-/-} mice. The pattern of connective tissue invasion also differed: cells in wild-type mice infiltrated as individually penetrating cells whereas in PAI-1^{-/-} mice they did so as a well-demarcated advancing front. Providing an alternative provisional matrix by impregnating sponges with a low concentration of collagen before implantation corrected the changes induced by PAI-1 deficiency. In conclusion, PAI-1 deficiency appears to affect fibrotic tissue formation in part by altering the provisional matrix that forms soon after tissue injury. (*Am J Pathol* 2003, 163:445–452)

Manipulation of the plasminogen system modulates the extent of fibrosis that follows bleomycin-induced lung injury. In particular, mice genetically deficient in plasminogen activator inhibitor-1 (PAI-1) are relatively protected from developing fibrosis after lung injury.^{1,2} Fibrosis is also reduced when plasminogen activation is enhanced in mice genetically engineered to express an inducible urokinase-type plasminogen activator (uPA) transgene

within the lung³ or after administration of an adenoviral vector containing a uPA gene.⁴ Conversely, fibrosis is worsened in mice having a constitutively active PAI-1 transgene¹ or in mice that are genetically deficient in plasminogen.⁵ Despite considerable efforts, it has been difficult to determine at what stage in the sequence of lung injury, repair, and fibrosis the plasminogen system is having a role. Evidence indicates that some of the protective effect from enhanced plasminogen activation occurs during the phase when the collagen content of the injured lung is increasing. In particular, administering an adenoviral vector containing a uPA transgene 21 days after bleomycin instillation reduces the amount of collagen accumulation when measured 7 days later.⁴

The possible mechanisms by which the plasminogen system modulates pulmonary fibrosis are multiple. As a generator of the broad-spectrum protease plasmin, the plasminogen system may assist with clearance of provisional matrices that form after tissue injury.⁶ Plasmin may also help remove extracellular material by activating other protease systems such as matrix metalloproteinases.^{7–9} The plasminogen system can further influence the fibrotic process by activating growth factors,^{10,11} altering cell adhesion and migration,^{12–14} and initiating intracellular signaling pathways.^{15,16} Determining which of these potential mechanisms is involved in protecting the lung against injury has been hampered by the complexities of the model systems being used. Because of these and other limitations, we wanted a simpler approach to study the role of the plasminogen system in the formation of fibrotic tissue. However, we also wanted to retain an *in vivo* experimental design to be certain that the fibrotic processes under investigation still relied on the biological responses of the intact animal. We therefore decided to examine the development of fibrotic tissue that is known to form within polyvinyl alcohol sponges that have been implanted subcutaneously into mice.^{17–20} This experimental approach allowed us to focus on one important aspect of the fibrotic process, namely the invasion of fibrotic tissue into tissue spaces and how PAI-1 deficiency alters this process.

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Materials and Methods

Animals

PAI-1^{-/-} mice were originally generated by Dr. Peter Carmeliet (University of Leuven, Leuven, Belgium)²¹ and backcrossed to C57BL/6 mice for eight generations. Age- and weight-matched wild-type C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA).

Sponge Implantation

Dry, sterile polyvinyl alcohol sponges (Ivalon Surgical Products, Eudora, KS) were cut into rectangles each weighing ~25 mg. Before implantation, each sponge was hydrated with phosphate-buffered saline (PBS). For the collagen impregnation experiments, sponges were expanded with 2.7 mg/ml of type I rat tail collagen (BD Biosciences, Bedford, MA) before implantation. Mice were anesthetized with 50 µg/g body weight pentobarbital, and their hair was clipped from the dorsal aspect of their trunk. After sterilizing the skin with betadine, two 1-cm transverse incisions were made, one on each side of the spine. Using a blunt forceps, a subcutaneous pocket was made cephalad from the incision and a sponge was sterilely inserted. Wound edges were approximated with a surgical stainless steel clip. At various times after sponge implantation, animals were sacrificed by CO₂ inhalation and the sponges removed.

Hydroxyproline Assay

After removal from the animal, any adherent tissue was stripped from the surface of the sponge. The sponges were minced in 1.0 ml of sterile H₂O using fine scissors. Next, 1.0 ml of concentrated HCl was added, and the samples were hydrolyzed at 120°C for 24 hours in a sealed glass tube.²² The solutions were then filtered through 45-µm pore filters. In a 96-well plate, 5 µl of each sample was mixed with 5 µl of citrate/acetate buffer (238 mmol/L citric acid, 1.2% glacial acetic acid, 532 mmol/L sodium acetate, and 85 mmol/L sodium hydroxide), after which 100 µl of chloramine-T solution (0.282 g chloramine-T added to 16 ml citrate/acetate buffer, 2.0 ml of *n*-propanol, and 2.0 ml H₂O) was added. After incubating the mixture for 30 minutes at room temperature, 100 µl of Ehrlich's solution (2.5 g of Ehrlich's reagent added to 9.3 ml of *n*-propanol and 3.9 ml of 70% perchloric acid) was added, and the samples were again incubated for 30 minutes at 65°C. Absorbance of each sample was measured at 550 nm. A standard curve was generated for each experiment using known concentrations of reagent hydroxyproline (Sigma Chemical Co., St. Louis, MO).

Histology, Invasion Area, and Vessel Density

After CO₂ euthanasia of the mice, the sponges were excised leaving the overlying skin attached. The sponges were then fixed in 10% neutral-buffered formalin overnight. Each sponge was then cut in half vertically, trans-

ferred to 70% ethanol, and embedded in paraffin. Five-µm sections were cut from the inner face of the cut blocks and stained with Masson's trichrome stain (Sigma Chemical Co.). For morphometric analysis, an entire cross-section of each sponge was visualized using an Eclipse E600 Nikon light microscope, and the image was stored digitally (Spot RT Slider camera and software; Diagnostic Instruments, Sterling Heights, MI). Using image processing software (Image-Pro Plus v4.0 for Windows; Media Cybernetics, Silver Springs, MD), the total sponge cross-sectional area and the area of the sponge that had been infiltrated by fibrotic tissue was measured. To quantify vessel density, photomicrographs were taken of day 28 sponges from eight random locations within the fibrotic tissue of each sponge using a ×40 objective lens. Image processing software (ImageJ; Scion Corp., Frederick, MD) was used to measure the area occupied by vessels within the fibrotic tissue at the eight sites within each sponge, and the average for each sponge was calculated. The investigator who captured the images and measured vessel areas was blinded to genotype. Vessel density was expressed as the percentage of cross-sectional area occupied by vessels within fibrotic regions.

Immunohistochemistry

Tissue sections were deparaffinized with xylene and rehydrated with graded alcohol steps. Sections were blocked with normal rabbit serum and then incubated with a 1:500 dilution of primary goat anti-mouse fibrin antiserum (Nordic Immunology, Tilburg, The Netherlands). Control slides were treated with nonimmune goat serum (1:500 dilution) in place of primary antibody. After washing the sections, bound antibody was detected using biotinylated rabbit anti-goat immunoglobulin and the Vectastain Elite ABC kit (both from Vector Laboratories, Burlingame, CA) using Nova Red as a peroxidase substrate (Sigma Chemical Co.).

Data Presentation and Statistical Analysis

Results are expressed as mean ± SEM. Rates of hydroxyproline accumulation or rates of fibrotic tissue invasion were compared by constructing least squares lines and comparing slopes according to the method of Zar²³ (Prism; GraphPad Software, San Diego, CA). Comparisons with *P* values of less than 0.05 were considered statistically significant.

Results

Collagen Content in Sponges

Hydroxyproline content was used as a measure of collagen accumulation within the implanted sponges. Hydroxyproline content of sponges from wild-type mice began increasing after 7 days and accumulated more rapidly thereafter (Figure 1). In the PAI-1^{-/-} mice, the rate of accumulation of collagen was significantly less relative to wild-type mice (*P* = 0.0002). At all time points

Figure 1. Kinetics of hydroxyproline accumulation in polyvinyl alcohol sponges. Sponges were implanted subcutaneously into wild-type or PAI-1^{-/-} mice and removed after various time intervals. The sponges were homogenized and the hydroxyproline content was measured. The hydroxyproline content in sponges from wild-type mice (●) increased more rapidly than it did in sponges from PAI-1^{-/-} mice (○). Data are expressed as mean ± SEM, *n* = 6 to 8, *P* = 0.0002.

studied, there was more hydroxyproline in sponges implanted into wild-type than PAI-1^{-/-} mice.

Histology and Immunohistochemistry

To provide a visual correlate to the hydroxyproline data, sponges were removed from mice and examined microscopically. At low magnification, the solid polyvinyl alcohol compound that makes up the sponge was seen as a labyrinth of pink-staining material within the rectangular shaped cross-sections (Figure 2A). Blue-staining mouse skin was seen overlying the top of the sponge. When viewed at higher magnification, sponges removed from wild-type mice on day 7 were found to contain a fine

fibrillar network of material that was seen coursing between the bubbly appearing, pink-staining polyvinyl alcohol matrix (Figure 2B). In addition, a sparse infiltrate that could be identified on higher magnification as neutrophils and mononuclear cells was seen scattered evenly throughout the sponge (data not shown). Immunohistochemical staining of day 7 sponges from wild-type mice showed that the fibrillar network contained an abundant amount of fibrin(ogen) (Figure 2C; control antibody, Figure 2D). In contrast, sponges removed at 7 days from PAI-1^{-/-} mice either lacked the fibrillar network entirely or had a markedly decreased amount of it (Figure 2E). Immunohistochemical staining for fibrin(ogen) detected little if any positive staining material in the PAI-1^{-/-} sponges (Figure 2F). Similar to wild-type mice, a sparse inflammatory cell infiltrate was present.

At day 21, microscopic examination showed that sponges removed from wild-type mice were progressively invaded by fibrotic tissue (Figure 3A). The infiltrate contained spindle-shaped cells typical of fibroblasts, capillary vessels containing red blood cells, and extracellular matrix material. The fibrous strands of the matrix stained blue in these Masson's trichrome-stained sections indicating the presence of mature collagen fibers. Although these same elements were present within sponges from PAI-1^{-/-} mice (Figure 3B), the pattern of infiltration was different. In wild-type mice, the cells at the advancing front of the fibrotic tissue appeared to percolate into the fibrin-rich provisional matrix as individual cells (Figure 3C). In the PAI-1^{-/-} mice, the advancing margin of fibrotic tissue was seen as a sheet of tissue that was distinctly demarcated from the open spaces within the central areas of the sponge (Figure 3D).

Figure 2. Photomicrographs of sections of sponges removed from wild-type and PAI-1^{-/-} mice 7 days after implantation. A low-magnification view of a trichrome-stained section of sponge removed from a wild-type mouse (A) shows the maze of pink-staining polyvinyl alcohol material. A higher magnification view (B) of the sponge interior from a wild-type mouse shows a sparse inflammatory cell infiltrate and a pink-staining fibrillar provisional matrix. Immunohistochemistry using an anti-mouse fibrin(ogen) antibody (C) or control antibody (D) shows that the fibrillar material stains positively (red-brown) for fibrin(ogen). Sections of sponges removed from PAI-1^{-/-} mice at 7 days show that the interior contains a sparse inflammatory cell infiltrate, but is devoid of the fibrillar network when viewed either with trichrome-staining (E) or anti-fibrin(ogen) immunostaining (F). Scale bars: 500 µm (A); 50 µm (B-F).

Figure 3. Photomicrographs of trichrome-stained sponge sections removed from wild-type and PAI-1^{-/-} mice after 21 days. Areas of sponge that have been infiltrated with fibrotic tissue in wild-type (A) and PAI-1^{-/-} (B) mice show similar findings with spindle-shaped cells, capillary vessels containing red blood cells, and blue-stained collagen fibrils coursing between the pink-stained polyvinyl alcohol material. The margin of the advancing fibrotic tissue in wild-type mice (C) shows individual cells entering the provisional matrix (from **top left** to **bottom right**) whereas in PAI-1^{-/-} mice (D), the advancing front appears as a sheet of cells (from **top left** to **bottom right**). Scale bars, 50 μ m.

Measurement of the Extent of Fibrotic Tissue Infiltration

To quantify the rate of fibrotic tissue invasion, the percentage of cross-sectional area of the sponge that was infiltrated by fibrotic tissue was measured on low-power photomicrographs (Figure 4). The rate of invasion was significantly slower in the PAI-1 null mouse sponges when compared to that of the wild-type mice ($P = 0.005$). In the PAI-1^{-/-} sponges, the invasion of fibrotic tissue progressed until day 21 but then remain stable from day 21 to 28. In contrast, the degree of invasion continued to increase in the sponges from wild-type animals through day 28.

Clearance of the Early Fibrin-Rich Matrix

As reported above, the fibrin-rich matrix that was seen within sponges from wild-type mice at day 7 was strikingly absent in sponges from PAI-1^{-/-} mice. To investigate this absence of provisional matrix, sponges were studied at earlier time points. Immunohistochemical examination of these sponges showed that a fibrin matrix was formed within sponges from PAI-1^{-/-} mice by 1 day after implantation (Figure 5A; control antibody Figure 5B),

but was subsequently removed by 4 days (Figure 5C). In wild-type mice, the fibrin-rich matrix that was evident by day 1 persisted at days 2, 4 (data not shown), and 7 (Figure 2C).

Figure 4. Kinetics of fibrotic tissue invasion of sponges implanted into wild-type and PAI-1^{-/-} mice. Sponges were implanted subcutaneously into wild-type or PAI-1^{-/-} mice and removed after various time intervals. Low-power photomicrographs were obtained of Masson trichrome-stained sections and the depth of fibrosis was measured and expressed as the fraction of total sponge area occupied by fibrotic tissue. Sponges implanted into wild-type mice (●) accumulated fibrotic tissue more rapidly than sponges placed into PAI-1^{-/-} mice (○). Data are expressed as mean $\pm$ SEM, $n = 4$ to 6, $P = 0.005$.

Figure 5. Photomicrographs of anti-fibrin(ogen)-immunostained sponge sections removed from PAI-1^{-/-} mice. Sponges harvested from mice 1 day after implantation show a fibrillar material that stained positively (red-brown) with the anti-fibrin(ogen) antibody (**A**) but not with the control antibody (**B**). Sponges removed from PAI-1^{-/-} mice after 4 days show that the staining with anti-fibrin(ogen) antibody is lost (**C**). Scale bars, 50 μ m.

Vessel Formation

The capillary vessels that formed within the fibrotic tissue appeared similar in sponges from wild-type and PAI-1^{-/-} mice. To assess this quantitatively, the vessel density within fibrotic tissue was measured by determining the percentage of cross-sectional area representing vessels on tissue sections obtained on day 28. There was no statistically significant difference ($P = 0.89$, t -test, $n = 6$ per group) in vessel density between wild-type mice ($2.41 \pm 0.39\%$, mean $\pm$ SEM) and PAI-1^{-/-} mice ($2.49 \pm 0.40\%$).

Fibrotic Tissue Formation within Type I Collagen-Impregnated Sponges

The impressive loss of the fibrin-containing provisional matrix in sponges from PAI-1^{-/-} mice suggested that this lack of structural support might limit the penetration of fibrotic tissue into sponges in PAI-1^{-/-} mice. To investigate this possibility, we formed a loose type I collagen matrix within sponges before implantation and then measured the rates of collagen accumulation and fibrotic tissue invasion. Similar to the data reported in Figure 1, the hydroxyproline content of noncollagen-impregnated sponges increased less rapidly after 7 days in PAI-1^{-/-} mice compared to wild-type mice (Figure 6) ($P = 0.02$). However, if a loose collagen matrix was formed within the sponge before implantation (containing $40.5 \pm 1.9 \mu\text{g}$ hydroxyproline/sponge), the rate of subsequent hydroxyproline accumulation was similar in PAI-1^{-/-} and wild-type mice ($P = 0.63$). To determine whether the kinetics of fibrotic tissue invasion were also altered by impregnating the sponges with type I collagen, the fraction of sponge area occupied by fibrotic tissue was measured at various times after implantation (Figure 7). Similar to the effect on hydroxyproline accumulation, the addition of type I collagen to the sponges before implantation eliminated the difference in rate of invasion between PAI-1^{-/-} and wild-type mice ($P = 0.94$). Also, the advancing front of fibrotic tissue in PAI-1^{-/-} mice (Figure 8) appeared similar to that seen within sponges lacking preformed collagen that had been implanted into wild-type mice (Figure 2C). The sharp demarcation between fibrotic tissue and sponge interior was lost.

Discussion

To investigate the effects of PAI-1 deficiency on the development of fibrosis, we used polyvinyl alcohol sponges so that we could control variables better than we could by using the lung as the target organ. These sponges have the additional advantage of providing a more reproducible fibrotic stimulus than what occurs in most of the lung fibrosis models. In the bleomycin model, for example, there is considerable heterogeneity in the amount and distribution of injury and subsequent fibrosis.^{4,24} Also, the subcutaneously implanted sponges can be easily extracted from the mice permitting access to pure fibrotic tissue for histological or biochemical analyses. Their location in the subcutaneous space provides an opportunity during future studies to inject solutions into the sponges and determine the effects of possible agonists or inhibitors of the fibrotic process.

Importantly, we found that the fibrotic process in sponges shares many features with pulmonary fibrosis. For example, implantation of sponges leads to fibrin accumulation within the interstices of the sponge similar to

Figure 6. Kinetics of hydroxyproline accumulation in polyvinyl alcohol sponges containing a preformed type I collagen matrix. Sponges were prepared containing either PBS or a type I collagen matrix and then implanted subcutaneously into wild-type or PAI-1^{-/-} mice. The sponges were removed at various time intervals and the hydroxyproline content measured. As seen in Figure 1, the hydroxyproline content of PBS-containing sponges accumulated more rapidly in wild-type mice (●) than in PAI-1^{-/-} mice (○) ($P = 0.02$). However, if the sponges were impregnated with a small amount of type I collagen before implantation, the rate of accumulation of hydroxyproline in sponges from PAI-1^{-/-} mice (□) was similar to that of wild-type mice (■) ($P = 0.63$). Data are expressed as mean $\pm$ SEM, $n = 4$.

Figure 7. Kinetics of fibrotic tissue invasion into sponges impregnated with type I collagen before implantation into wild-type and PAI-1^{-/-} mice. Sponges were prepared containing either PBS or type I collagen and then implanted subcutaneously into wild-type or PAI-1^{-/-} mice. The fraction of sponge cross-sectional area that was invaded by fibrotic tissue was measured as in Figure 4. The impregnation of type I collagen into sponges before implantation eliminated the difference in the rate of fibrotic tissue invasion in sponges between wild-type (●) and PAI-1^{-/-} mice (○) ($P = 0.94$). Data are expressed as mean $\pm$ SEM, $n = 3$ to 4.

what occurs in the alveolar space during pulmonary fibrosis in humans and bleomycin-induced lung fibrosis in animals.²⁵ In both circumstances, there is the invasion of mesenchymal cells and generation of a collagen-containing extracellular matrix.^{17–20,24} Furthermore, PAI-1 deficiency led to reduced collagen accumulation in the polyvinyl alcohol sponge model similar to what occurs in the lung after inflammatory injury. Although the effects of modulating the plasminogen system on lung fibrosis have been consistent in the various models,^{1–5} the mechanisms by which this effect occurs remain uncertain.

By isolating certain aspects of the fibrotic process using the polyvinyl alcohol sponge model, we have been able to clarify some of the effects of PAI-1 deficiency on the development of fibrosis. In particular, we found that PAI-1 deficiency delayed the invasion of tissue spaces by

Figure 8. Photomicrograph of a collagen-impregnated sponge implanted into PAI-1^{-/-} and removed after 21 days. Trichrome-stained sponge sections show that the advancing front of fibrotic tissue in sponges impregnated with collagen before implantation into PAI-1^{-/-} mice has an appearance similar to that of nonimpregnated sponges inserted into wild-type mice (see Figure 3C). Scale bar, 50 μ m.

fibrotic tissue and delayed the accumulation of collagen. A similar delay in the generation of fibrotic tissue in the lung after injury might be beneficial by allowing more time for reconstitution of damaged alveolar tissues before irreversible scarring occurs. Histological examination of the sponges also revealed important qualitative and quantitative differences in the pattern of cellular infiltration between PAI-1^{-/-} and wild-type mice. In sponges implanted into PAI-1^{-/-} mice, the leading edge of the fibrotic tissue appeared as a sharply demarcated border separating it from the open spaces within the center of the sponge. With time, the leading edge advanced into the sponge as if it were being built up layer by layer. In wild-type mice, the leading edge of the fibrotic tissue, although still discernible, was less distinct with individual spindle-shaped cells penetrating the sponge interior.

Although there are many mechanisms that could explain the different patterns of fibrotic tissue invasion in wild-type and PAI-1^{-/-} mice, a simple explanation is suggested by the differences in behavior of the provisional fibrin-rich matrix that forms within the sponges shortly after implantation. In both wild-type and PAI-1^{-/-} mice, a fine network of acellular material formed within the sponge interstices as early as 1 day after insertion. The material stained prominently for fibrin(ogen) suggesting that it was formed from leakage of plasma-derived fluid into the sponges shortly after implantation. The striking loss of this material within a few days in PAI-1^{-/-} mice is likely because of an increased generation of plasmin resulting from the absence of its major inhibitor of activation, ie, PAI-1. We have observed this phenomenon previously in injured mouse lungs where fibrin is removed more rapidly in mice with enhanced plasminogen activation.^{1,2}

The accelerated removal of the provisional matrix in the PAI-1^{-/-} mice led to the hypothesis that the loss of a scaffold on which the advancing fibrotic tissue invades might explain the histological and biochemical differences that were observed between wild-type and PAI-1^{-/-} mice. This hypothesis was tested by providing an alternative matrix on which cells might migrate into the sponge interior. When a loose collagen matrix was formed within sponges before implantation in PAI-1^{-/-} mice, the delays in fibrotic tissue invasion and collagen accumulation were corrected. Admittedly, the invasion of cells into collagen differs from invasion into a fibrin-rich matrix. Different cellular adhesion molecules and proteases are likely used to penetrate fibrin *versus* collagen. However, our experimental results still support the hypothesis that loss of a provisional matrix might be an important mechanism by which PAI-1^{-/-} mice develop delayed fibrosis after tissue injury. This conclusion is supported by work done by Drew and colleagues²⁶ who implanted porous tubing subcutaneously into control and fibrinogen-deficient mice. In the absence of fibrinogen, the interior of the tubing was not invaded by fibrotic tissue.

The apparent role of the provisional matrix in the fibrotic process of the sponge model has implications for understanding the development of fibrosis in damaged organs. The majority of evidence has shown a tight cor-

relation between the generation of active plasmin and protection of the lung from fibrosis. However, the proteolytic targets of the active enzymes in the plasminogen system are still uncertain. Previous work has shown that the critical substrate is not exclusively fibrin, because fibrinogen-deficient mice still develop pulmonary fibrosis after bleomycin-induced injury.^{2,27} Because plasmin has a rather broad proteolytic specificity, there are many other proteins that could be cleaved by accelerated plasminogen activation. Potential candidates are fibronectin, laminin, proteoglycans, or other matrix proteins that could serve as a platform and anchor for fibrotic tissue formation.⁶

Although a major effect of PAI-1 deficiency is an accelerated removal of the provisional matrix, other actions of the plasminogen system may be involved in limiting fibrosis. These include activation of latent growth factors such as transforming growth factor- β ¹¹ and hepatocyte growth factor/scatter factor;¹⁰ activation of other proteolytic cascades including matrix metalloproteinases;⁷⁻⁹ participation in cell adherence and migration via interactions of PAI-1^{-/-} with vitronectin that influences binding of integrins and the urokinase-type plasminogen activator receptor to extracellular matrix;¹²⁻¹⁴ and initiation of intracellular signaling cascades via the urokinase receptor and partnering molecules.^{15,16}

PAI-1 deficiency has been shown to decrease angiogenesis in a number of experimental situations. For example, the absence of PAI-1 inhibited vessel invasion into murine fibrosarcomas²⁸ and into Matrigel implants.²⁹ Angiogenesis was also greatly reduced within collagen matrices embedded subcutaneously into mice.^{30,31} Furthermore, the amount of choroidal angiogenesis induced by laser photocoagulation in the eyes of PAI-1^{-/-} mice was less than that of wild-type mice.³² Contrary to these previously published articles, the experiments reported here show that capillary vessels can form normally within the connective tissue in sponges implanted into PAI-1^{-/-} mice. That a situation exists in which angiogenesis is unaltered by PAI-1 deficiency is not surprising because blood vessels form normally during fetal development and postnatal growth in PAI-1^{-/-} mice.²¹ For those experimental conditions in which PAI-1 is required for angiogenesis, the PAI-1 appears to be functioning in its protease-inhibiting role^{33,34} and/or in its ability to inhibit interactions between vitronectin and the urokinase receptor or $\alpha_v\beta_3$ integrin.³⁴ Apparently, neither of these functions of PAI-1 is required for blood vessel formation in the fibrotic tissue that forms within the sponges. Modulators of protease activity and cell adhesion other than PAI-1 must be sufficient to allow vessels to form.

The effects on fibrosis caused by modulation of the plasminogen system are not restricted to the lung or to the implanted sponges used in these studies. Other investigators have shown that manipulations of the plasminogen system can influence fibrosis in such diverse conditions as immune-mediated glomerulonephritis,³⁵ antigen-induced arthritis,³⁶ chemical injury to the pleural space,³⁷ surgical trauma to the eye,³⁸ liver cirrhosis,³⁹ obstructive uropathy in the kidney,⁴⁰ and vascular fibrosis.⁴¹ The generic effects of PAI-1 deficiency on the

accelerated removal of provisional matrices as demonstrated in the sponge model may have direct relevance to these and other fibrotic conditions.

In summary, we have found that PAI-1 deficiency delays the development of fibrosis within tissue spaces. The results of the current study strongly suggest that one likely mechanism is the accelerated removal of the fibrin-rich provisional matrix that forms and serves as a scaffold on which mesenchymal cells invade to form the fibrotic scar.

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