Plasminogen activator inhibitor-1 in acute hyperoxic mouse lung injury.

C Barazzone, … , J D Vassalli, A P Sappino

J Clin Invest. 1996;98(12):2666-2673. https://doi.org/10.1172/JCI119089.

Research Article

Hyperoxia-induced lung disease is associated with prominent intraalveolar fibrin deposition. Fibrin turnover is tightly regulated by the concerted action of proteases and antiproteases, and inhibition of plasmin-mediated proteolysis could account for fibrin accumulation in lung alveoli. We show here that lungs of mice exposed to hyperoxia overproduce plasminogen activator inhibitor-1 (PAI-1), and that PAI-1 upregulation impairs fibrinolytic activity in the alveolar compartment. To explore whether increased PAI-1 production is a causal or only a correlative event for impaired intraalveolar fibrinolysis and the development of hyaline membrane disease, we studied mice genetically deficient in PAI-1. We found that these mice fail to develop intraalveolar fibrin deposits in response to hyperoxia and that they are more resistant to the lethal effects of hyperoxic stress. These observations provide clear and novel evidence for the pathogenic contribution of PAI-1 in the development of hyaline membrane disease. They identify PAI-1 as a major deleterious mediator of hyperoxic lung injury.

Plasminogen Activator Inhibitor-1 in Acute Hyperoxic Mouse Lung Injury

Constance Barazzone,*‡ Dominique Belin,‡ Pierre-François Piguet,‡ Jean-Dominique Vassalli,§ and André-Pascal Sappino‖
**Department of Pediatrics, ‡Department of Pathology, §Department of Morphology, and ‖Department of Medicine, University of Geneva, Geneva, Switzerland*

Abstract

Hyperoxia-induced lung disease is associated with prominent intraalveolar fibrin deposition. Fibrin turnover is tightly regulated by the concerted action of proteases and antiproteases, and inhibition of plasmin-mediated proteolysis could account for fibrin accumulation in lung alveoli. We show here that lungs of mice exposed to hyperoxia overproduce plasminogen activator inhibitor-1 (PAI-1), and that PAI-1 upregulation impairs fibrinolytic activity in the alveolar compartment. To explore whether increased PAI-1 production is a causal or only a correlative event for impaired intraalveolar fibrinolysis and the development of hyaline membrane disease, we studied mice genetically deficient in PAI-1. We found that these mice fail to develop intraalveolar fibrin deposits in response to hyperoxia and that they are more resistant to the lethal effects of hyperoxic stress. These observations provide clear and novel evidence for the pathogenic contribution of PAI-1 in the development of hyaline membrane disease. They identify PAI-1 as a major deleterious mediator of hyperoxic lung injury. (*J. Clin. Invest.* 1996. 98:2666–2673.) Key words: hyperoxia • fibrin • proteases • fibrinolysis • plasmin

Introduction

Premature babies and adults suffering from respiratory distress syndrome (RDS)[1] often require artificial ventilation with high oxygen concentration to maintain adequate blood oxygenation. The detrimental consequences of hyperoxia on lung tissues are widely recognized: high concentrations of oxygen are toxic for most cellular constituents of the lung and induce irreversible damage. Hyperoxic lung injury is characterized by the prominent intraalveolar deposition of fibrin and cell debris, the classical feature of hyaline membrane disease (1). Fibrin accumulation in alveolar spaces is considered to play a major role in respiratory failure and in the subsequent development of fibrosis (2). Degradation of fibrin is mediated by plasmin, the production of which is tightly regulated by the concerted actions of proteases and antiproteases: the plasminogen activators (PAs) and their specific inhibitors (PAIs). By controlling the rate of plasmin formation, the balance of PAs and PAIs ensures fibrinolytic homeostasis in both vascular and extravascular compartments. During pathological processes the production of PAs and PAIs is frequently altered, leading either to excessive plasmin-catalyzed proteolysis as observed in neoplasia, or, alternatively, to impaired plasmin formation as reported in lipopolysaccharide-induced injury, sepsis, or renal disorders (3–6). Clinical studies have demonstrated reduced plasmin-mediated proteolysis in bronchoalveolar lavages (BAL) of patients with adult RDS (7, 8). Plasminogen activator inhibitor type 1 (PAI-1) gene expression is upregulated in lungs of rats exposed to hyperoxia, suggesting that the PA/plasmin system in lung tissues is altered (9). Using a murine model that recapitulates the morphologic and functional alterations of oxygen toxicity observed in humans (10), we have explored the effects of hyperoxia on the PA/plasmin system in lung tissues. We demonstrate here that hyperoxia-induced fibrin accumulation is associated with a reduction of plasmin-mediated proteolysis in alveolar spaces and that this decrease is attributable to a selective increase of PAI-1 production. Furthermore, we show that mice genetically deficient for PAI-1 fail to exhibit alveolar fibrin accumulation in response to hyperoxia. These mice are protected against the lethal effects of hyperoxia. Altogether, our observations directly document the pathogenic role of antiprotease production in alveolar spaces exposed to high oxygen concentration.

Methods

Mice. C57BL/6 mice were purchased from Iffa Credo (Labresle, France) and bred for three generations in our animal facility. SV129 mice were obtained from BRL (Basel, Switzerland) and used only for survival experiments. PAI-1 −/−, PAI-1 +/+ wild-type, tissue-type plasminogen activator (tPA) −/−, and urokinase-type plasminogen activator (uPA) −/− (SV129 and C57BL/6 mixed background) mice were kindly provided by P. Carmeliet (University of Leuven, Belgium) and bred in our animal facility (11, 12). Experiments were performed with 2-mo-old male mice weighing 20–25 grams.

Hyperoxia exposure. Mice were placed in a sealed Plexiglas chamber with a minimal in- and outflow, providing six to seven exchanges per hour of the chamber volume and maintaining CO_2 levels

Address correspondence to Dr. C. Barazzone, Departments of Pediatrics and Pathology, Centre Médical Universitaire, 1, rue Michel Servet, 1211 Genève 4, Switzerland. Phone: 41-22-372-57-57; FAX: 41-22-702-57-46; E-mail: Constance.Barazzone@medecine.unige.ch

Received for publication 8 July 1996 and accepted in revised form 11 October 1996.

1. *Abbreviations used in this paper:* α2-AP, α2-antiplasmin; BAL, bronchoalveolar lavage; PAI-1, plasminogen activator inhibitor type 1; PN-I, protease-nexin I; RDS, respiratory distress syndrome; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; W/D, lung wet over dry weight.

J. Clin. Invest.

0021-9738/96/12/2666/08 $2.00
Volume 98, Number 12, December 1996, 2666–2673

below 1%. Animals were exposed to 100% O_2 for different periods of time. O_2 and CO_2 levels were measured daily with an O_2 and CO_2 analyzer (Engström, Sweden) as described (10). Mice exposed to room air in the same chamber served as controls. Food and water were available ad libitum. This study protocol was approved by the ethical committee on animal experiments (Office Vétérinaire Cantonal of Geneva).

Assessment of pulmonary leakage. Mice exposed to hyperoxia or to room air were injected intravenously 2 h before time of killing with 200 μl of Evans blue (10 mg/ml in sterile saline solution; Fisher Scientific, Pittsburgh, PA). Blood was rinsed from lung vessels by injecting the right ventricle with 5 ml saline; lungs were then inflated by instilling 5% formaline (in 80% ethanol) into the trachea. When the alveolo-capillary barrier is altered by endothelial injury, the protein-adsorbed dye diffuses from the vascular bed into the alveolar space and colors the whole lung blue. In control animals, dye staining remains within blood vessels. Pulmonary edema was determined by weighing both lungs at autopsy and after drying the tissue at 80°C until two weights, 24 h apart, remained unchanged. The lung wet over dry weight (W/D) was normalized to the initial body weight.

Histological analyses. Mice were bled through the abdominal aorta under light anesthesia and the thorax was opened to abolish the negative intrathoracic pressure. Lungs were fixed by instilling formaline in the trachea with a hydrostatic pressure of 20 cm. Transhilar horizontal sections were embedded in paraffin and processed for light microscopy. Cryostat tissue sections were prepared from lungs instilled with tissue-TEK OCT and frozen immediately with methylbutane into liquid nitrogen.

PAI-1 immunohistochemical detection was performed on 5-μm cryostat tissue sections fixed with paraformaldehyde. After preincubation with normal goat serum, sections were incubated overnight at room temperature with rabbit anti–mouse PAI-1 serum (final dilution 1:200; provided by D. Loskutoff, La Jolla, CA). Controls were performed with rabbit nonimmune IgG at equivalent concentration (50 μg/ml) (provided by G. Gabbiani, Department of Pathology, Geneva). Fibrin(ogen) immunohistochemical detection was performed on 7-μm paraffin-embedded formalin-fixed sections postfixed in cold acetone. After preincubation with normal goat serum, sections were incubated with rabbit anti–mouse fibrinogen IgGs, which recognize fibrinogen and fibrin-derived products (final dilution 1:500). Controls were performed with the corresponding preimmune serum at the same dilution. Both sera were provided by D. Simon (Boston, MA). All tissue sections were incubated with alkaline phosphatase–conjugated goat anti–rabbit IgGs (1:20; Dako, Copenhagen, Denmark) for 30 min and revealed with the fast red colorimetric kit (Dako) for 5 min. Immunostainings were performed in three different mice for each condition. The immunohistochemical sections were scanned with a high sensitivity Photonic Science Coolview color camera (Carl Zeiss, Oberkochen, Germany) through an Axiophot photomicroscope (Zeiss) equipped with a plan apochromate × 40/1.40 oil immersion objective. The camera was connected to a 486DX2/66 Intel PC and the images were captured using the software package Image Access (release 2.04; IMAGIC, Bildverarbeitung AG, Glattbrug, Zürich, Switzerland). 10 randomly selected fields were analyzed for each antiserum-labeled section and 5 for the control staining of an adjacent section. Image analysis was carried out with a Zeiss image analyzer using the software package KS 400 (release 2.0; Kontron Electronik, GmbH, Eching, Germany). For each condition an index expressed in arbitrary units was calculated. This index integrates the relative area and the mean intensity of the immunostaining. The mean intensity was given by the mean value in HLS (hue, lightness, and saturation) model. The results were expressed after having subtracted the values of the control staining.

RNA analyses. Lungs from saline-washed mice were dissected, frozen immediately in liquid nitrogen, and stored at −80°C. After homogenization in guanidinium/thiocyanate, total RNA was isolated by cesium chloride centrifugation as described previously (13). uPA, tPA, PAI-1, PAI-2, α2-antiplasmin (α2-AP), and protease-nexin I (PN-I) probes were prepared as described (14–18) and transcribed in vitro using α ^{32}P-labeled UTP (specific activity 400 Ci/mmol; Amersham International, Buckinghamshire, United Kingdom). RNase protection assays were performed as described (19). Quantitation was achieved by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA) using the Image Quant software (Molecular Dynamics, Inc.). Five different samples were analyzed for each condition and results are expressed as mean increase compared with control mice±SD.

Protein analyses. BAL was performed by instilling into the trachea 3 ml of saline solution containing 0.2 M EDTA, 5 mM 2-iodoacetamide (Sigma Chemicals, St. Louis, MO), and 250 U/ml aprotinin (Bayer, Switzerland); BAL fluid was recovered immediately on ice under negative hydrostatic pressure and the supernatant was collected after centrifugation. BAL and tissue protein concentration was determined by Bradford's method (20). Tissue protein extraction and zymography were performed as described (15, 21). To dissociate protease–antiprotease complexes, NH_4OH (4%) was added to samples 30 min before electrophoresis. Quantitation was made by scanning the zymographies with the Image Quant software. Histological zymograms were performed on lung cryostat tissue, as described (14). Slides were incubated at 37°C in a humidified chamber and were allowed to develop for 2–6 h. Parallel experiments were carried out in the absence of plasminogen or in the presence of 1 mM amiloride, a selective inhibitor of uPA catalytic activity (22).

Statistical analysis. For each parameter measured or calculated, the values for all animals in an experimental group were averaged and the SD of the mean was calculated. The significance of differences between the values of an experimental group and those of the control group was determined with the unpaired Student's *t* test. Survival parameters were analyzed using the Kaplan-Meier test. Significance levels were set at $P < 0.05$.

Results

Hyperoxia induces intraalveolar fibrin deposition. C57/BL6 mice subjected to continuous hyperoxia developed progressive pulmonary lesions that became lethal within 4–5 d. Histologic alterations of the pulmonary parenchyma were first detectable after 72 h of exposure. Endothelial cells displayed morphologic evidence of damage, while septal and alveolar edema was apparent. More pronounced lesions were observed after 90 h of hyperoxia. Alveolar walls then appeared thickened and were lined with severely damaged epithelial cells. Concomitantly, large amounts of extracellular material containing fibrin were observed in the alveolar spaces (Fig. 1); this feature is characteristic of diffuse hyaline membrane deposition.

Hyperoxia reduces plasmin-mediated proteolysis in the alveolar compartment. Histological zymographies performed on lung tissue sections of hyperoxia-exposed mice revealed a decreased uPA-mediated proteolytic activity throughout areas corresponding to the alveolar compartment, while zones of tPA-mediated proteolysis (corresponding to vascular structure) were unaffected (not shown). Whole lung tissue protein extracts of air-breathing and hyperoxia-exposed mice were analyzed by SDS-PAGE and zymography. In control mice, two types of plasminogen-dependent proteolytic activities were detected, corresponding to uPA and tPA, respectively (Fig. 2 *A*). After 90–96 h of hyperoxia, a slight decrease in uPA-mediated catalytic activity was observed, while tPA-mediated activity remained unchanged (Fig. 2 *A*); high molecular weight PA–PAI complexes became visible after longer development of the zymograms. In contrast, protein extracts of BAL from mice exposed to hyperoxia showed a marked reduction in uPA activ-

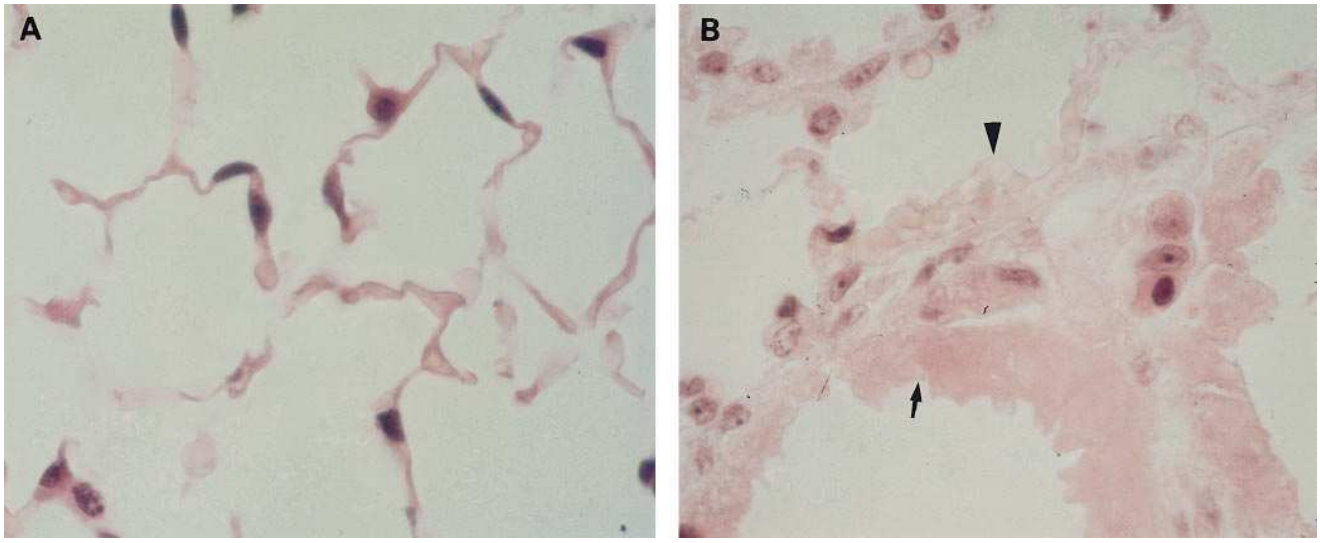

Figure 1. Lung histology of mice exposed to air (*A*) or to 100% O_2 (*B*) for 90 h. Sections were stained with hematoxylin and eosin. The arrow points to hyaline membranes. Note the presence of red blood cells into alveoli (*arrowhead*). ×100.

ity ($P = 0.02$), and to a lesser extent in tPA activity ($P = 0.06$) (Fig. 2 *B*). Abundant high molecular weight PA–PAI complexes appeared during hyperoxia ($P = 0.03$). When BAL specimens were incubated with NH_4OH before electrophoresis to dissociate complexes formed between uPA or tPA and their inhibitors, uPA and tPA-mediated activities were completely restored and were more abundant in hyperoxic than in control samples (Fig. 2 *C*). The analysis of BAL by reverse zymography revealed the presence of increased levels of PAI-1 in hyperoxia exposed mice. Thus, the complexes with uPA and tPA can be attributed, at least in part, to PAI-1. We also analyzed the plasma fibrinolytic activity of mice submitted to the same conditions and found no difference in uPA or tPA proteolytic activity between control and hyperoxic-treated mice (not shown).

These findings indicate that uPA-mediated proteolysis is reduced in the alveolar compartment exposed to hyperoxia and raise the possibility that this reduction of fibrinolytic potential can be ascribed to the concomitant presence of increased amounts of PAI(s).

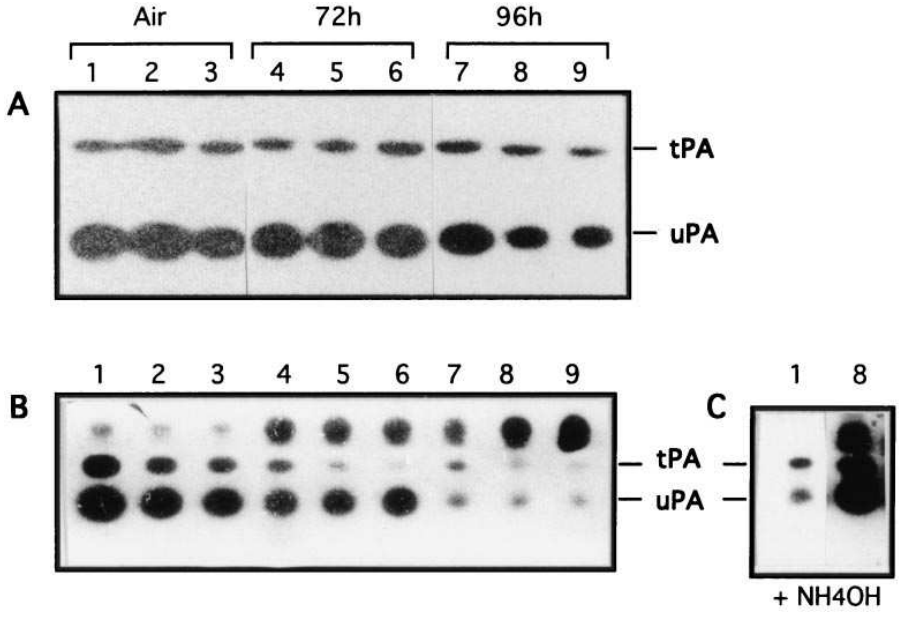

Figure 2. Zymographic analysis of PA activities in total lung (*A*) and BAL (*B* and *C*). Lanes *1–3*: control air-breathing mice; lanes *4–9*: mice exposed to 100% O_2 for 72 h (lanes *4–6*) and 90–96 h (lanes *7–9*). (*A*) 20 μg of total lung protein from one animal was loaded onto each lane. Photograph was taken after 16 h of incubation at 37°C. The migration of tPA and uPA was determined with purified standard proteins electrophoresed in adjacent lanes. (*B*) 20 μg of protein recovered in BAL from individual mice was loaded onto each lane. Note the marked reduction in uPA-mediated activity ($P < 0.05$) and to a lesser extent in tPA activity (P = NS) after 90–96 h of hyperoxia. High molecular weight PA–PAI complexes are more prominent in BAL of hyperoxic animals ($P < 0.05$). The photograph was taken after 40 h of incubation at 37°C. (*C*) Samples of BAL from air-breathing (lane *1*) and 90–96 h hyperoxia-exposed mice (lane *8*) were treated with NH_4OH before electrophoresis to dissociate PA–PAI complexes. The photograph was taken after 24 h of incubation. Proteolytic activity was restored in the hyperoxia-exposed sample (lane *8*).

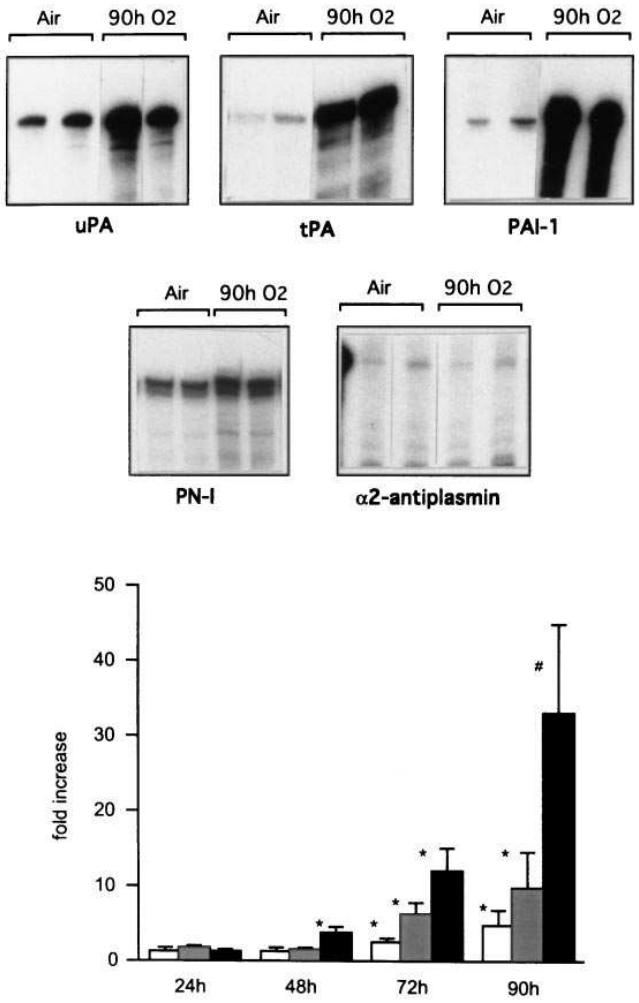

Figure 3. Quantitation of uPA, tPA, and PAI-1 mRNAs in murine lungs. Mice were exposed to air or to 100% O_2 for the indicated times. 10 μg of total lung RNA was hybridized to ^{32}P-labeled cRNA probes. RNase-resistant hybrids were analyzed after separation in urea/polyacrylamide gels. PN-I and α-2 antiplasmin mRNAs were used as controls, since they do not increase during hyperoxia. (*Bottom*) Quantitation of mRNA levels was performed with a Molecular Dynamics PhosphorImager. Values represent means of five animals±SD ($n = 3$ for 24 h of hyperoxia) and are expressed as fold-increase compared with controls set as 1. $^{*}P < 0.05$ versus control, $^{\#}P < 0.001$ versus control. *White bars*, uPA; *gray bars*, tPA; *black bars*, PAI-1.

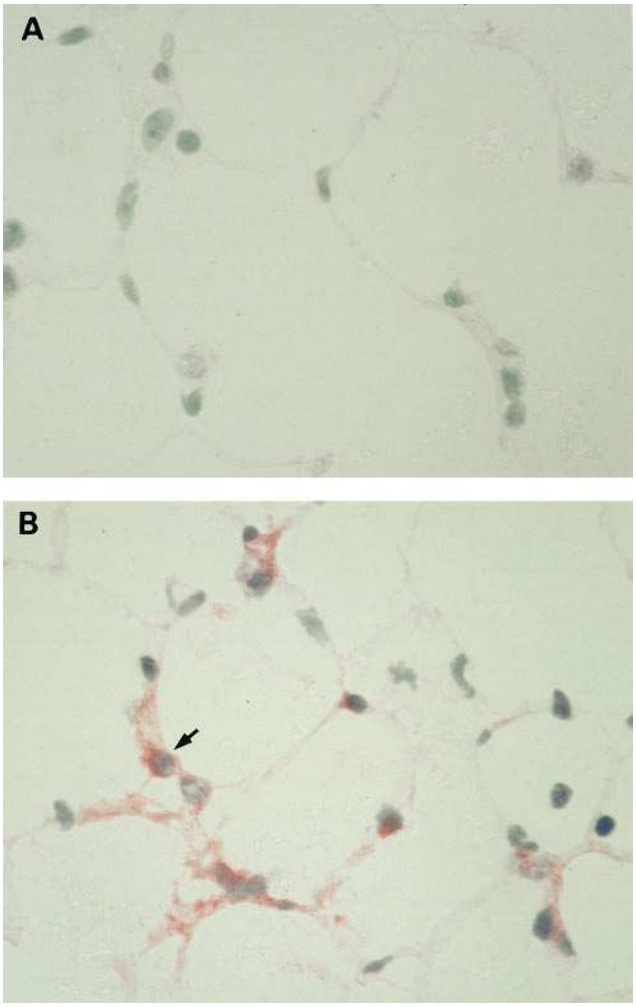

Figure 4. Immunohistochemical localization of PAI-1 in murine lungs. Cryostat tissue sections were stained with a rabbit antimurine PAI-1 (1:200) as described in Methods. Micrographs of representative fields are presented. No PAI-1 could be detected in the lungs of air-breathing mice (*A*). Quantitation analysis of the immunostaining revealed a sixfold increase in PAI-1 immunoreactivity in animals exposed to hyperoxia compared with air-breathing animals ($P < 0.01$). The arrows point to PAI-1 containing alveolar septal cells in the lung of a mouse exposed to hyperoxia for 84 h (*B*). ×60. Identical results were obtained with three different animals.

Hyperoxia preferentially upregulates PAI-1 gene expression in lung tissues. The presence of uPA, tPA, PAI-1, PAI-2, α2-AP, and PN-I mRNAs was assessed by RNase protection analysis of total lung RNA extracted from air-breathing and hyperoxia-exposed mice. Lungs exposed to 90–96 h of hyperoxia showed a 30-fold increased PAI-1 mRNA content and only a 4–9-fold increase in uPA and tPA mRNA contents, when compared with lungs from air-breathing mice (Fig. 3). Increased PAI-1 mRNA accumulation was already apparent after 48 h of hyperoxia, while increased uPA and tPA mRNA content was only detectable after 72 h of hyperoxia. In contrast, no modulation of PAI-2, PN-1, or α2-AP mRNA levels was observed. PAI-1 protein was assessed by immunohistochemistry. PAI-1 immunostaining was observed in alveolar septa and in some alveolar cells which, according to their localization, might correspond to type II epithelial cells (Fig. 4). Quantitation of the immunochemical reaction indicated a sixfold higher level of PAI-1 in hyperoxic mice as compared with air-breathing animals ($P < 0.05$).

These investigations show that PAI-1 gene expression is markedly induced during hyperoxic lung injuries and that the protein is secreted in the alveolar compartment; the increased antiprotease production precedes intraalveolar fibrin accumulation.

PAI-1 contributes to the pathogenesis of hyperoxic lung injuries. To determine whether the increased PAI-1 gene expression directly contributes to alveolar fibrin accumulation and to the pathogenesis of hyaline membrane disease, we placed mice genetically deficient for PAI-1 under the same hyperoxic conditions.

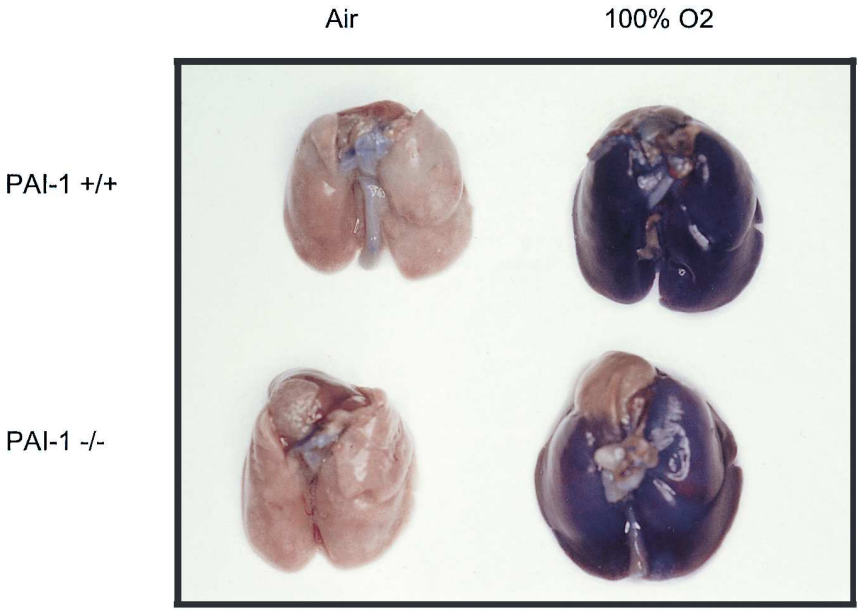

Figure 5. Macroscopic visualization of vascular leakage in the lungs of PAI-1 −/− and PAI-1 +/+ (C57/BL6 × 129) mice. Air-breathing mice (*left*) and mice exposed to 100% O_2 for 84 h (*right*) were injected intravenously with 200 μl of Evans Blue 2 h before time of killing. Hyperoxic lungs of both PAI-1 −/− and PAI-1 +/+ mice show an intense and homogeneous blue color, indicating a similar alveolar leakage in both strains, whereas no leakage is observed in air-breathing lungs.

Histological examination of lung tissues revealed striking differences in the response to hyperoxia. Lungs of PAI-1 −/− and PAI-1 +/+ mice submitted to 96 h of hyperoxia differed significantly. PAI-1 −/− mice exhibited minimal alterations at this time; septal thickening was only observed focally and no fibrin deposits could be found in alveolar spaces. In contrast, lung microscopic examination of uPA −/− and tPA −/− strains revealed septal thickening, prominent epithelial cell injuries, and intraalveolar fibrin deposition. These alterations were similar to those observed in PAI-1 +/+ mice, but they

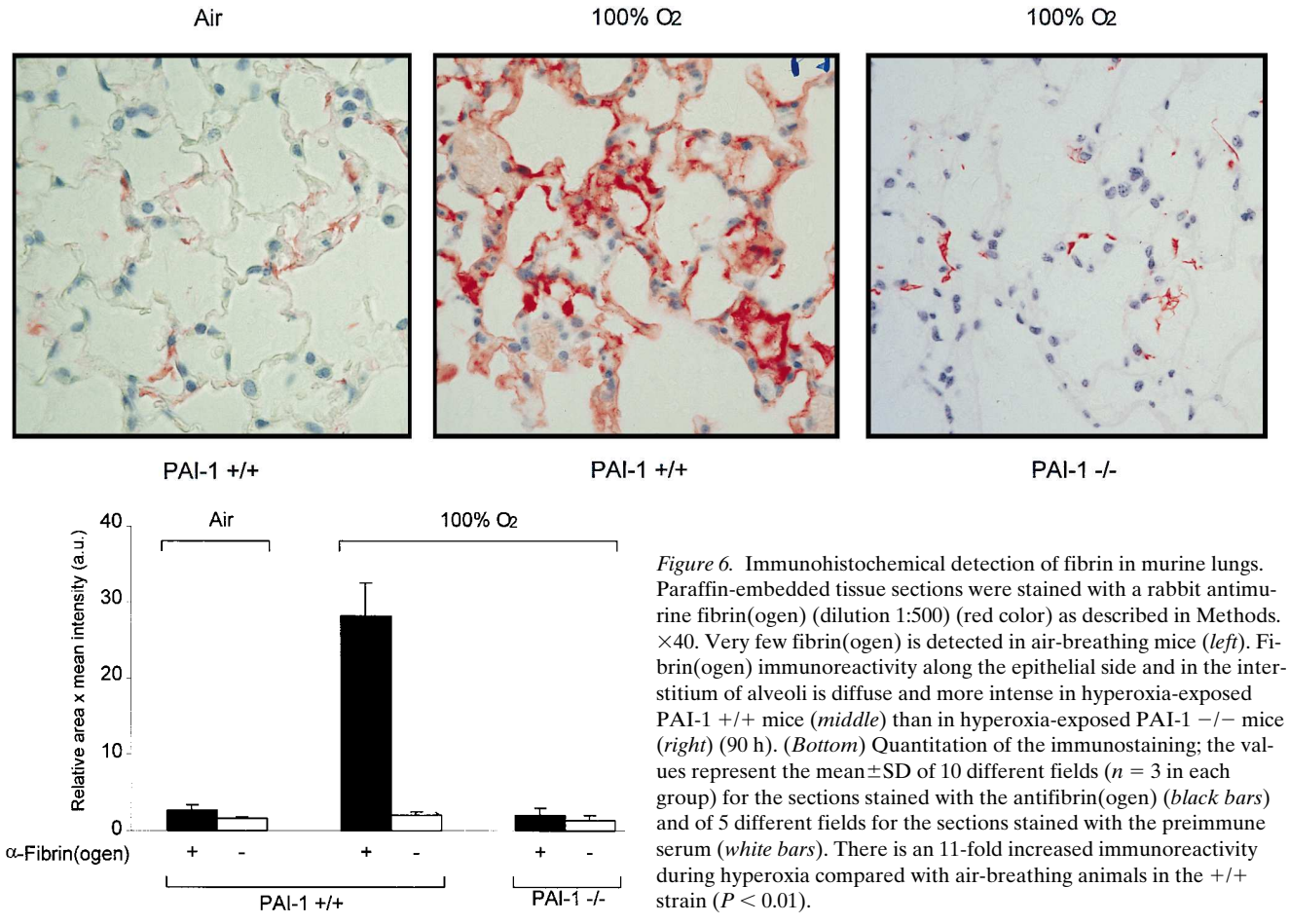

Figure 6. Immunohistochemical detection of fibrin in murine lungs. Paraffin-embedded tissue sections were stained with a rabbit antimurine fibrin(ogen) (dilution 1:500) (red color) as described in Methods. ×40. Very few fibrin(ogen) is detected in air-breathing mice (*left*). Fibrin(ogen) immunoreactivity along the epithelial side and in the interstitium of alveoli is diffuse and more intense in hyperoxia-exposed PAI-1 +/+ mice (*middle*) than in hyperoxia-exposed PAI-1 −/− mice (*right*) (90 h). (*Bottom*) Quantitation of the immunostaining; the values represent the mean±SD of 10 different fields ($n = 3$ in each group) for the sections stained with the antifibrin(ogen) (*black bars*) and of 5 different fields for the sections stained with the preimmune serum (*white bars*). There is an 11-fold increased immunoreactivity during hyperoxia compared with air-breathing animals in the +/+ strain ($P < 0.01$).

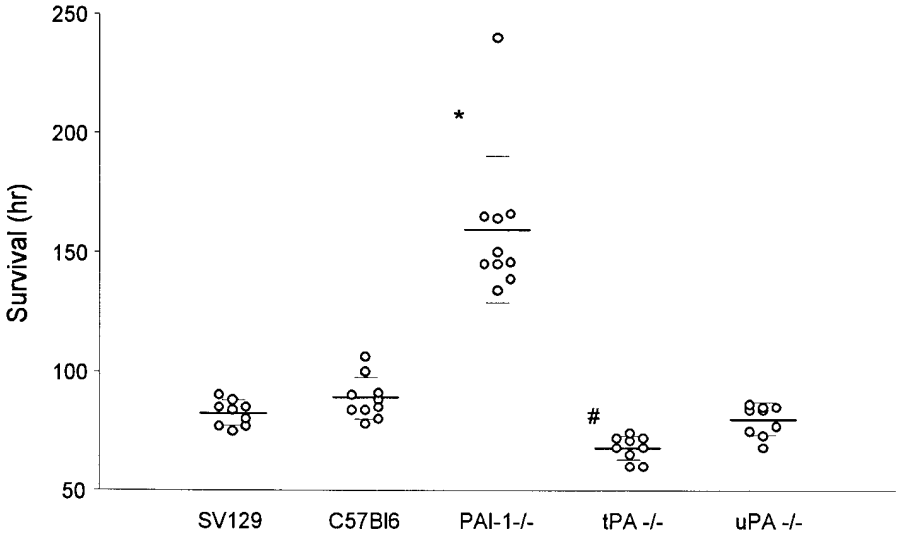

Figure 7. Survival time of mice exposed to hyperoxia (100% O_2). PAI-1 −/− mice ($n = 10$) survive significantly longer than C57/BL6 ($n = 10$) or SV129 mice ($n = 9$) ($^*P < 0.0001$). tPA- and uPA-deficient mice (−/−) have a genetic background similar to PAI-1 −/− mice. Their survival in 100% O_2 is similar (uPA −/− strain) or slightly reduced (tPA −/− strain) compared with that of the two parent strains ($^\#P < 0.05$).

appeared earlier (48–56 h). Finally, zymographic analysis of BAL protein extracts showed that tPA- and uPA-mediated fibrinolytic activities were maintained during hyperoxia in PAI-1 −/− mice (not shown).

Vascular leakage is considered to be an early event of hyperoxic injury. Protection conferred by PAI-1 deficiency could result from increased fibrinolysis either in the vascular space or in the alveolar compartment. To distinguish between these two possibilities, we first assessed vascular leakage by intravenous injection of Evans blue. Hyperoxia provoked similar pulmonary leakage in PAI-1 −/− and PAI-1 +/+ mice, suggesting that the endothelium of PAI-1 −/− mice is not protected against hyperoxic injury (Fig. 5). We also analyzed BAL protein content and W/D ratio (normalized to initial body weight), in PAI-1 +/+ and −/− mice. Both were increased during hyperoxia exposure, but they were not significantly different between the two animal groups. In air-breathing PAI-1 +/+ and −/− mice, BAL protein content was 0.17 and 0.14 mg/ml, respectively; in hyperoxia-exposed mice (90 h), BAL protein content was 1.37 and 1.05 mg/ml, respectively ($n = 5$). After 90 h of hyperoxia, (W/D)/body weight ratio was 0.27±0.04 (PAI-1 +/+, $n = 7$) vs. 0.25±0.04 (PAI-1−/−, $n = 6$). We then analyzed fibrin deposition by immunochemistry (Fig. 6). An 11-fold increase was detected in the alveoli of hyperoxia-exposed PAI-1 +/+ mice ($P < 0.01$, vs. air-breathing mice), whereas minimal fibrin deposition occurred along alveolar walls of PAI-1 −/− mice.

We finally compared the survival of PAI-1 −/− mice with that of C57/BL6 and SV129 mice, from which they were derived (11). PAI-1 −/− mice survived significantly longer than C57/BL6 and SV129 mice: mean survival±SD was 159±10 h vs. 94±9 and 82±5 h ($P < 0.0001$) (Fig. 7). Since the genetic background of PAI-1 −/− mice is not well defined, we also analyzed the survival of genetically comparable mice which are PAI-1 +/+, but either uPA −/− or tPA −/− (12). tPA −/− mice died at 68±5 h and uPA −/− at 79±6 h (Fig. 7). The survival of tPA −/− mice is significantly shorter than of the parent strains ($P < 0.05$).

Taken together these observations show that PAI-1 deficiency protects from the lethality of hyperoxia by preventing intraalveolar fibrin accumulation.

Discussion

Fibrin is involved in a wide spectrum of extravascular remodeling processes. In wound repair, localized fibrin deposition secures the restoration of tissue integrity through its mechanical and biological properties. In certain pathological conditions, such as in lung disorders, excessive fibrin formation has been postulated to play a pivotal role in the generation of organ damage (23). Acute lung injuries are often associated with fibrin-enriched intraalveolar exudates, and fibrin deposition in air spaces impairs gas exchanges. Fibrin also favors the subsequent development of fibrosis by stimulating the proliferation of collagen-synthesizing fibroblasts (2, 24, 25). Thus, inappropriate fibrin accumulation appears to be a pathogenic event in both acute and chronic phases of respiratory failure. Fibrin accumulation integrates the combined influences of capillary leak, intraalveolar coagulation, and fibrinolysis, which is controlled by the balance between PAs and PAIs.

During hyperoxia the synthetic potential of lung tissue for uPA, tPA, and PAI-1, three components of the PA/plasmin system, is increased. The upregulation of PAI-1 mRNA accumulation being most dramatic, the balance may be tilted towards reduced plasmin formation. While overall uPA- and tPA-mediated plasmatic and lung tissular proteolytic activities are similar in hyperoxic and air-breathing animals, striking differences are observed in corresponding BAL samples. uPA and tPA activities are markedly reduced, most likely as a result of complex formation with PAI-1. Histological zymographies of lung sections confirm that the hyperoxia-induced decrease in lung proteolytic activity affects primarily uPA-catalyzed activity associated with alveolar structures, whereas tPA activity was essentially unaffected. Thus, the perturbation of the plasmin-mediated proteolytic balance during hyperoxia appears to involve the alveolar compartment, where it may play a decisive role in favoring fibrin accumulation. Our results are in accordance with previous work exploring the fibrinolytic system in experimental and clinical interstitial lung diseases (4, 8, 26, 27). BAL fibrinolytic activity is decreased in RDS and has been attributed to PAI-1 expression (7, 8). Similarly, impaired fibrinolysis correlates with fibrin accumulation in animal models of hyperoxia (28).

The cellular origins of lung uPA, tPA, and PAI-1 remain to be determined. uPA may be synthesized by pneumocytes and/or resident monocytes/macrophages, while tPA is expressed predominantly by endothelial cells (21, 29, 30). Immunohistochemical findings suggest an epithelial origin for PAI-1 production, but resident monocytes/macrophages, fibroblasts, and endothelial cells may also contribute to its synthesis (9, 26, 31, 32). The upregulation of PAI-1 synthesis may be a direct consequence of hyperoxia, as shown in vitro (9). It could also be triggered by the alveolar presence of fibrin(ogen), which was reported recently to induce PAI-1 synthesis by rat lung fibroblasts (33). Our results do not support this hypothesis, since PAI-1 gene expression appears earlier than fibrin deposition.

Genetically deficient mice provide unique tools to discriminate between causal and coincidental changes in gene expression. Using mice made deficient in PAI-1 by gene targeting, we have demonstrated here that this antiprotease plays a deleterious role in acute lung hyperoxia. Indeed, PAI-1–deficient mice exposed to hyperoxia have persistent fibrinolytic activity in their alveoli, they fail to develop intraalveolar fibrin deposits, and they are relatively resistant to the lethal effect of hyperoxia. PAI-1 −/− mice are not completely protected against all toxic effects of hyperoxia, however. After 96 h of exposure to hyperoxia, lungs of PAI-1 −/− mice show morphologic and functional signs of endothelial cell damage, including vascular leakage. These results suggest that endothelial cell lesions precede impaired alveolar fibrinolysis and therefore are an upstream event in the development of hyaline membrane disease. The analysis of tPA-deficient mice supports the functional relevance of altered plasmin-mediated fibrinolysis in hyperoxic lung injury. These mice develop severe pulmonary lesions earlier than wild-type mice in response to hyperoxia. Why tPA-deficient mice are more susceptible to hyperoxia than uPA-deficient mice is not clear. tPA activity seems to be less affected than uPA activity during hyperoxia. Therefore, tPA could prevent fibrin deposition at early times during hyperoxia, while its absence in tPA-deficient mice would accelerate fibrin-induced alveolar damage. Conversely, uPA, being present essentially within the alveolar compartment, may not be crucial until alveolar leakage and subsequent fibrin deposition is present, after 72 h of hyperoxia. Several authors have reported that decreased fibrinolytic activity associated with experimental and clinical pulmonary lesions is a good predictive marker for the subsequent development of fibrosis (28, 34, 35). In accordance with this, it has been shown recently that PAI-1–mediated inhibition of fibrinolysis influences the severity of bleomycin-induced chronic lung fibrosis (36). Our findings provide strong evidence for a causal link between decreased PA activity in BAL and lethal lung injury in acute hyperoxic disease.

Fibrin is one of the most potent inactivators of surfactant (37); thus, the presence of intraalveolar fibrin can be highly detrimental during the course of RDS in premature babies. Surfactant administration is now a standard treatment to prevent hyaline membrane disease (38). If intraalveolar fibrin accumulation could be avoided or at least limited, this may improve the beneficial effect of surfactant administration, not only during the acute phase but also for the reparative phase. Intratracheal administration of uPA has been suggested as a means to improve the clinical course of chronic respiratory diseases (39). The identification of PAI-1 as a major contributor to the pathogenesis of hyperoxia-induced lung lesions offers an alternative pharmacological strategy to restore intraalveolar fibrinolysis. Agents that modulate PAI-1 synthesis, or agents that could prevent interaction of this antiprotease with its target enzymes, such as noninhibitory PAI-1 analogues, might help control the deleterious effect of PAI-1 in lung diseases.

Acknowledgments

We thank P. Carmeliet and J. Huarte for providing the mice; M.-L. Bochaton-Piallat for her assistance on image analysis; A.F. Junod, M.M. Philippeaux, F. Tacchini-Cottier, P. Vassalli, and A.L. Wohlwend for helpful and stimulating discussions; M. Vuagnat for help with statistical analysis; C. Combépine, Y. Donati, M. Redard, A.F. Rochat, N. Sappino, V. Sciretta, J. Stalder, and C. Vesin for their excellent technical assistance; and J.C. Rumbeli and E. Denking for photographic work.

C. Barazzone, D. Belin, P.F. Piguet, J.-D. Vassalli, and A.-P. Sappino are supported by the Fonds national suisse de la recherche scientifique. C. Barazzone was also supported by the Foundations Sandoz and Sir Jules Thorn.

References

1. Crapo, J.D., B.E. Barry, H.A. Foscue, and J. Shelburne. 1980. Structural and biochemical changes in rat lungs occurring during exposure to lethal and adaptive doses of oxygen. *Am. Rev. Respir. Dis.* 122:123–143.

2. Basset, F.V., V.J. Ferrans, P. Soler, P. Takemura, Y. Fukuda, and R.G. Crystal. 1986. Intraluminal fibrosis in interstitial lung disorders. *Am. J. Pathol.* 122:443–461.

3. Danø, K., P.A. Andreasen, J. Grøndhal-Hansen, P. Kristensen, L.S. Nielsen, and L. Shriver. 1985. Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer Res.* 44:139–266.

4. Sawdey, M.S., and D.J. Loskutoff. 1991. Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. *J. Clin. Invest.* 88:1346–1353.

5. Suffredini, A.F., P.C. Harpel, and J.E. Parillo. 1989. Promotion and subsequent inhibition of plasminogen activation after administration of intravenous endotoxin to normal subjects. *N. Engl. J. Med.* 320:1165–1172.

6. Moll, S., P.A. Menoud, T. Fulpius, Y. Pastore, S. Takahashi, L. Fossati, J.D. Vassalli, A.P. Sappino, and S. Izui. 1995. Induction of plasminogen activator inhibitor type 1 in murine lupus-like glomerulonephritis. *Kidney Int.* 48: 1459–1468.

7. Bertozzi, P., B. Astedt, L. Zenzius, K. Lynch, F. Lemaire, W. Zapol, and H. Chapman. 1990. Depressed bronchoalveolar urokinase activity in patients with adult respiratory distress syndrome. *N. Engl. J. Med.* 322:890–897.

8. Idell, S., K.K. James, T.R. Martin, J. McLarty, and R. Maunder. 1991. Trended abnormalities of fibrin turnover in evolving adult respiratory distress syndrome. *Am. J. Lung Cell. Mol. Physiol.* 5:L240–L248.

9. White, J.E., M. Ryan, M.F. Tsan, and P.J. Higgins. 1993. Hyperoxic stress elevates p52(PAI-1) mRNA abundance in cultured cells and adult rat pulmonary tissue. *Am. J. Physiol.* 265:L121–L126.

10. Barazzone, C., F. Tacchini-Cottier, C. Vesin, A. Rochat, and P.F. Piguet. 1996. Hyperoxia induces platelet activation and lung sequestration: an event dependent on tumor necrosis factor-α and CD11a. *Am. J. Respir. Cell Mol. Biol.* 15:107–114.

11. Carmeliet, P., L. Kieckens, L. Schoonjans, B. Ream, A. Van Nuffelen, G. Prendergast, M. Cole, R. Bronson, D. Collen, and R.C. Mulligan. 1993. Plasminogen activator inhibitor-1 gene-deficient mice. I. Generation by homologous recombination and characterization. *J. Clin. Invest.* 92:2746–2755.

12. Carmeliet, P., L. Schoonjans, L. Kieckens, B. Ream, J. Degen, R. Bronson, R. De Vos, J.J. van der Oord, D. Collen, and R.C. Mulligan. 1994. Physiological consequences of loss of plasminogen activator gene function in mice. *Nature (Lond.).* 368:419–424.

13. Busso, N., D. Belin, C. Failly-Crépin, and J.D. Vassalli. 1986. Plasminogen activators and their inhibitors in a human mammary cell line (HBL-100). *J. Biol. Chem.* 261:9309–9315.

14. Sappino, A.P., J. Huarte, J.D. Vassalli, and D. Belin. 1991. Sites of synthesis of urokinase and tissue-type plasminogen activators in the murine kidney. *J. Clin. Invest.* 87:962–970.

15. Sappino, A.P., R. Madani, J. Huarte, D. Belin, J.Z. Kiss, A.L. Wohlwend, and J.D. Vassalli. 1993. Extracellular proteolysis in the adult murine brain. *J. Clin. Invest.* 92:679–685.

16. Belin, D., A.L. Wohlwend, W.D. Schleuning, E.K. Kruithof, and J.D. Vassalli. 1989. Facultative polypeptide translocation allows a single mRNA to encode the secreted and the cytosolic form of plasminogen activator inhibitor 2. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3287–3294.

17. Menoud, P.-A., N. Sappino, M. Boudal-Khoshbeen, J.-D. Vassalli, and A.-P. Sappino. 1996. The kidney is a major site of α_2-antiplasmin production. *J. Clin. Invest.* 97:2478–2484.

18. Vassalli, J.D., J. Huarte, D. Bosco, A.P. Sappino, N. Sappino, A. Velardi, A. Wohlwend, H. Ernø, D. Monard, and D. Belin. 1993. Protease-nexin I as an androgen-dependent secretory product of the murine seminal vesicle. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:1871–1878.

19. Belin, D. 1994. The use of riboprobes for the analysis of gene expression. *In* Protocols for Gene Analysis. Vol. 31. A.J. Harwood, editor. Humana Press Inc., Towota, NJ. 257–272.

20. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

21. Vassalli, J.D., J.M. Dayer, A. Wohlwend, and D. Belin. 1984. Concomitant secretion of prourokinase and of plasminogen activator-specific inhibitor by stimulated macrophages. *J. Exp. Med.* 159:1653–1668.

22. Vassalli, J.D., and D. Belin. 1987. Amiloride selectively inhibits the urokinase-type plasminogen activator. *FEBS Lett.* 214:187–191.

23. Idell, S., K.K. James, B. Levine, B.S. Schwartz, N. Manchanda, R.J. Maunder, T.R. Martin, J. McLarty, and S. Fair. 1989. Local abnormalities in coagulation and fibrinolytic pathways predispose to alveolar fibrin deposition in the adult respiratory distress syndrome. *J. Clin. Invest.* 84:695–705.

24. Kuhn, C., and J.A. McDonald. 1991. The roles of the myofibroblasts in idiopathic pulmonary fibrosis. Ultrastructural and immunohistochemical features of sites of active extracellular synthesis. *Am. J. Pathol.* 138:1257–1265.

25. Mc Donald, J.A. 1991. Idiopathic pulmonary fibrosis. A paradigm for lung injury and repair. *Chest.* 99:87S–93S.

26. Olman, M.A., N. Mackman, C.L. Gladson, K.M. Moser, and D.J. Loskutoff. 1995. Changes in procoagulant and fibrinolytic gene expression during bleomycin-induced lung injury in the mouse. *J. Clin. Invest.* 96:1621–1630.

27. Biemond, B.J., M. Levi, H. TenCate, T. Van der Poll, H.R. Büller, C.E. Hack, and J.W. TenCate. 1995. Plasminogen activator and plasminogen activator inhibitor I release during experimental endotoxaemia in chimpanzees: effect of intervention in the cytokine and coagulation cascade. *Clin. Sci.* 88:587–594.

28. Idell, S., A. Kumar, K.B. Koenig, and J.J. Coalson. 1994. Pathways of fibrin turnover in lavage of premature baboons with hyperoxic lung injury. *Am. J. Respir. Crit. Care Med.* 149:767–775.

29. Gross, T., R. Simon, C. Kelley, and R. Sitrin. 1991. Rat alveolar epithelial cells concomitantly express plasminogen activator inhibitor-1 and urokinase. *Am. J. Physiol.* 260:L286–L295.

30. Loskutoff, D.J., and T.S. Edgington. 1977. Synthesis of a fibrinolytic activator and inhibitor by endothelial cells. *Proc. Natl. Acad. Sci. USA.* 74:3903–3907.

31. Chapman, H.A., X.L. Yang, L.Z. Sailor, and D.J. Sugarbaker. 1990. Developmental expression of plasminogen activator inhibitor type 1 by human alveolar macrophages. Possible role in lung injury. *J. Immunol.* 145:3398–3405.

32. Varani, J., R.G. Sitrin, and W. Hillegas. 1992. Expression of plasminogen activator and plasminogen activator inhibitor mRNA in human fibroblasts grown on different substrates. *Cytotechnology.* 9:157–162.

33. Hagood, J.S., M.A. Olman, J.A. Godoy, K.E. Rivera, and G.M. Fuller. 1996. Regulation of type I plasminogen activator inhibitor by fibrin degradation products in rat lung fibroblasts. *Blood.* 87:3749–3757.

34. Singhal, K.K., and L.A. Parton. 1996. Plasminogen activator activity in preterm infants with respiratory distress syndrome: relationship to the development of bronchopulmonary dysplasia. *Ped. Res.* 39:229–235.

35. Viscardi, R., K. Broderick, C.C. Sun, A.J. Yale-Loehr, A. Hessamfar, V. Taciak, K.C. Burke, K.B. Koenig, and S. Idell. 1992. Disordered pathways of fibrin turnover in lung lavage of premature infants with respiratory distress syndrome. *Am. Rev. Respir. Dis.* 146:492–499.

36. Eitzman, D.T., R.D. McCoy, X. Zheng, W.P. Fay, D. Ginsburg, and R.H. Simon. 1996. Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *J. Clin. Invest.* 97:232–237.

37. Seeger, W., G. Stoehr, H.D. Wolf, and H. Neuhof. 1985. Alteration of surfactant function due to protein leakage: special interaction with fibrin monomer. *J. Appl. Physiol.* 58:326–338.

38. Halliday, H.L. 1995. Overview of clinical trials comparing natural and synthetic surfactants. *Biol. Neonate.* 67:32–47.

39. Hart, D.A., P. Whidden, F. Green, J. Henkin, and D.E. Woods. 1994. Partial reversal of established bleomycin-induced pulmonary fibrosis by rh-urokinase in a rat model. *Clin. Invest. Med.* 17:69–76.