Enhanced fibrinolysis protects against lung ischemia–reperfusion injury

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Objective: Ischemia–reperfusion injury continues to plague the field of lung transplantation, resulting in suboptimal outcomes. In acute lung injury, processes such as ventilator-induced injury, sepsis, or acute respiratory distress syndrome, extravascular fibrin has been shown to promote lung dysfunction and the acute inflammatory response. This study investigates the role of the fibrinolytic cascade in lung ischemia–reperfusion injury and investigates the interplay between the fibrinolytic system and the inflammatory response.

Methods: Mice lacking the plasminogen activator inhibitor-1 gene (PAI-1 knock out, PAI-1 KO; and thus increased lysis of endogenous fibrin) and wild-type mice underwent in situ left lung ischemia and reperfusion. Fibrin content in the lung was evaluated by immunoblotting. Reperfusion injury was assessed by histologic and physiologic parameters. Proinflammatory mediators were measured in bronchoalveolar lavage fluid and plasma using enzyme-linked immunosorbent assays.

Results: Ischemia–reperfusion causes fibrin deposition in murine lungs. Less fibrin was seen in PAI-1 KO mice than in wild-type mice subjected to the same ischemia–reperfusion conditions. By histologic criteria, more evidence of ischemia–reperfusion injury was noted (thickening of the interstitium, cellular infiltration in the alveoli) in the wild-type than in PAI-1 KO mice. Physiologic parameters also revealed more ischemia–reperfusion injury in the wild-type than in PAI-1 KO mice. Cytokine and chemokines were elevated more in the wild-type group than the PAI-1 KO group.

Conclusions: Lung ischemia–reperfusion injury triggers fibrin deposition in the murine lungs and fibrin creates a proinflammatory environment. Preventing fibrin deposition may reduce ischemia–reperfusion injury and inflammation. This finding may lead to novel treatment strategies for ischemia–reperfusion.

Lung transplantation currently is the preferred treatment option for a variety of end-stage pulmonary diseases. Remarkable progress has occurred through refinement in technique and improved understanding of transplant immunology and microbiology. Despite improvements in preservation, ischemia–reperfusion injury (IRI) continues to result in unpredictable suboptimal outcomes in lung transplant recipients. The incidence of IRI ranges from 10% to 25% in most series,1,2 and there is mounting evidence that IRI is associated with an increased risk for the development of bronchiolitis obliterans.3,4

IRI is a form of acute lung injury. It is widely documented that increased coagulation and impaired fibrinolysis play an important role in the pathogenesis of the various forms of acute lung injury.5 Extravascular fibrin deposition has been shown to promote lung dysfunction, and furthermore intravascular thrombosis occurs in the acutely injured lung.5 Depression of fibrinolysis has been shown to increase detection of fibrin, and this results mostly through amplification of plasminogen activator inhibitor-1 (PAI-1), which is a 52-kDa serine protease inhibitor that serves as the major plasma inhibitor of tissue-type plasminogen activator and urokinase-type plasminogen activator. Hypoxia has been shown also to increase PAI-1 and promote pulmonary vascular fibrin deposition.7 Figure 1 details the fibrinolytic/PAI-1 pathway.

An important area of recent interest has been the interplay between coagulation and inflammation in acute lung injury, and much has been written on this topic in acute lung injury from adult respiratory distress syndrome, sepsis, and ventilator-induced lung injury.5 One concept that has emerged over the past decade is that coagulation and inflammation are linked and should not be considered different distinct pathways.5 Anticoagulants reduce inflammation and result in improved outcomes in animal models of sepsis and acute lung injury.5 Tissue fibrin is known to influence local alveolar inflammation in a variety of ways. Therefore, fibrinolysis may be useful for preventing inflammation during lung IRI.

This article examines the role changes in the fibrinolytic pathway in IRI by using PAI-1 knock-out (KO) mice.
in our mouse ischemia–reperfusion (IR) model. We try to investigate the association between fibrin deposition and the inflammatory response in our experimental model. We hypothesize that enhancing fibrin turnover will decrease inflammation and protect the lung from in our IRI mouse model.

METHODS

Animal Care

C57BL6 (wild-type; WT) and PAI-1 knock-out (PAI-1 KO; also on a C57BL6 background) male mice (Jackson Laboratory, Bar Harbor, Maine) (28–35 g) were used for all studies and received humane care in accordance with the “Principles of Laboratory Animal Care,” formulated by the National Society for Medical Research, and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health. The study protocol was reviewed and approved by the Animal Care and Use Committee at the University of Virginia before experimentation.

Experimental Protocol of IR

Three experimental groups (WT-sham, WT-IR, and PAI-1 KO IR) were compared using an in vivo left hilar clamp model. WT-IR and PAI-1 KO IR groups underwent 1 hour of left hilar clamping and closure but no hilar clamping. A minimum of 6 mouse lungs were studied per group (6 mice with 1 lung each). In this model of IR lung injury, mice are anesthetized with inhalational isoflurane, intubated, connected to a volume-controlled ventilator (Harvard Apparatus Co, South Natick, Mass), and ventilated with room air. Heparin (30 U/kg) is given via injection into the right external jugular vein. The left hilum is approached via a left thoracotomy. The left fourth rib is cut with a cautery pen at about 2 mm lateral to the sternum. A 6–0 Prolene polypropylene suture (Ethicon, Inc, Somerville, NJ) is placed around the left hilum and both ends are threaded out through a piece of polyethylene (PE)-10 tubing. The left hilum is occluded by pushing down the PE tubing while pulling tension on the suture. A small surgical clip is placed around the suture to keep the PE tubing in position. The left lung is rendered ischemic for 1 hour; reperfusion is then established by removing the PE tubing and the suture. The wound is suture-closed in layers. The mouse is allowed to awaken and recover in the cage. The WT-sham operated controls were subjected to the same perioperative protocols as the mice (ie, sedation, hydration, warming mattress); a left thoracotomy was performed with dissection of the hilum and then left thoracotomy site was closed.

In Situ Buffer-Perfused Mouse Lung Model (to Obtain Physiologic Parameters)

After 2 hours of reperfusion, one group of the mice in each set of experiments was reanesthetized and pulmonary function evaluated by an in situ buffer-perfused mouse lung system. The isolated perfused mouse lung system (Hugo Sachs Elektronik–Harvard Apparatus GmbH, March-Hugestten, Germany) was used as previously described by our laboratory.8 An advantage of this system is that the lungs are not removed from the chest cavity, but instead the whole animal is placed in the chamber with an open chest. In brief, the animal is anesthetized (pentobarbital sodium, 160 mg/kg, intraperitoneally) and placed on the heated operating table, and a tracheotomy is performed. The skin is incised in a median line from the abdomen to the neck. Positive-pressure ventilation is initiated at 120 breaths/min before the thorax is opened, and ventilation pressure is controlled so that a tidal volume of approximately 15 mL/kg is achieved with a positive end-expiratory pressure of 2 cm H2O. The abdominal wall is incised and the incision extended laterally on both sides to the lower extremities so that the vessels of the thigh become visible and accessible. The diaphragm was carefully removed and the thorax is opened by median sternotomy. The animals are exanguinated by a cut through the renal vein 30 seconds after injection of 50 units of heparin. Krebs–Henseleit buffer that contains 2% albumin, 0.1% glucose, and 0.3% HEPES is used for perfusing the lungs. The Krebs solution is prepared to mimic right ventricular blood (mixed venous blood) using a gas bubbling stone with titrated gases generating a pH of 7.40 to 7.45, a PO2 of 60 to 70 mm Hg, and a PCO2 of 50 to 60 mm Hg. The blood-perfused and isolated lung apparatus are maintained at 37°C. With the use of an operating microscope, the thymus is dissected cephalad exposing the great vessels. The pulmonary artery and left atrium are cannulated, and the perfusate flow is set to a final perfusion rate of 2 mL/min. The left ventricle is vented with a small incision at the apex of the heart. The mitral apparatus is carefully dilated and the left atrial cannula is passed through the mitral valve and into the left atrium. The placement of the pulmonary artery and left atrial cannulas is further confirmed by a pressure tracing generated.
by the Pulmody system (Hugo Sachs Elektronik). Lungs are allowed to equilibrate for a 10-minute stabilization period. The following parameters are measured and stored using the computer data acquisition software: (1) pulmonary compliance, (2) pulmonary artery pressure, and (3) airway resistance.

**Western Blot**

For these experiments, each mouse was injected with 20 units of heparin before being humanely killed. Immediately after harvest, the mouse’s left lung was placed in ice-cold homogenizing buffer (20 mmol/L Tris, 100 mmol/L NaCl, and 2.7 mg/mL heparin) and then homogenized by six 10-second rounds of homogenization. Tissue extracts were incubated with 0.32-unit of human plasmin (Sigma Chemical Co, St Louis, Mo) at 37°C for total 8 hours on a rotating shaker. After centrifugation, collected supernatants were transferred to a fresh tube and stored at −80°C for analysis. The solubilized protein was then loaded onto a sodium dodecylsulfate-polyacrylamide gel for electrophoresis after the concentration of total protein was adjusted. Transferred membranes were blocked with 5% nonfat skim milk for 2 hours and incubated with murine monoclonal antibody against fibrin (American Diagnostic, Stamford, Conn) for 16 hours at 4°C. After being washed three times with Tris-buffered saline with 0.05% Tween 20, the membranes were incubated with peroxidase-conjugated secondary antibody for 2 hours. The membrane was washed three times with Tris-buffered saline and developed using Amersham ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, United Kingdom).

**Histology**

Whole-lung tissue specimens were immediately fixed in 10% formalin. After 24 hours they were embedded in paraffin and then cut and stained with hematoxylin and eosin or stained by immunohistochemistry.

**Immunohistochemical staining.** The experiments were performed as described previously. A rat anti-mouse neutrophils antibody (AbD Serotec, Raleigh, NC) and a rat anti-mouse macrophage antibody (Mac-2, Accurate Chem, Westbury, NY) were used as primary antibodies. Alkaline phosphatase–conjugated anti-rat immunoglobulin G (Sigma) was used as secondary antibody. The signals were detected with Fast-Red Alkaline phosphatase–conjugated anti-rat immunoglobulin G (Sigma) was used as secondary antibody. The sections were counterstained lightly with hematoxylin for viewing negatively stained cells. The number of neutrophils and macrophages per high-power-field was assessed via immunohistochemical staining of peripheral lung sections. Six fields were counted per lung. Three individuals who count the positive stained cells were blinded to the condition being assessed. The average cell number was used for statistical analysis.

**Plasma.** The mice for blood collection in this experiment were not used for obtaining physiologic parameters using the in situ buffer-perfused mouse lung model. After the animal was humanely killed, the blood was collected by left arterial puncture (approximately 0.80–1 mL per mouse) into tubes containing 50 uL of 169 mmol/L ethylenediaminetetraacetic acid. Plasma was collected by centrifugation (15,000 g for 5 minutes) and stored at −80°C for later cytokine analyses.

**Bronchoalveolar lavage (BAL) fluid collection.** BAL fluid was collected by washing with 2 separate aliquots of 1 mL of Ca2+- and Mg2+-free Hanks balanced salt solution (Gibco BRL, Life Technologies, Inc, Grand Island, NY) through the trachea. The first wash was centrifuged and the BAL supernatant stored for cytokine and chemokine analysis. The second wash was centrifuged, and the cell pellet from the first wash was pooled with the cell pellet from the second.

**Cytokine and chemokine analysis.** Monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α), keratinocyte chemotaxtant (KC), and macrophage inflammatory protein-2 (MP-2) were chosen to be measured. All chemokine and cytokine measurements were performed simultaneously to reduce errors because of interassay variation.
FIGURE 3. Immunohistochemical staining of neutrophils and macrophages in mice lung. Cells stained red indicate neutrophils or migratory macrophage infiltration. All sections were counterstained lightly with hematoxylin for viewing negatively stained cells. A, D, and G are stained with anti-Mac-2 antibody, B, E, and H are stained with antineutrophils antibody and C, F, and I are stained with normal rat immunoglobulin G (IgG). All figures are 200× magnifications. The bar graph shows the analysis of positive immunostaining of neutrophils and macrophages in the left lung of sham, WT-IR, and PAI-1 KO IR groups. Data shown are the mean ± standard error values for each group.
extractions, we cannot determine from these experiments which compartment (intra-alveolar, intravascular, or extra-vascular) accounted for the decrease in fibrin deposition in PAI-1 KO mice. Additionally, it could be a combination of compartments.

**Histology and Immunohistochemistry**

The histologic samples of the left lung of WT-sham, WT-IR, and PAI-1 KO IR mice are shown in Figure 3. The interstitium of the left lung in WT-IR and PAI-1 KO IR mice appeared thicker with more cellularity and edema than that of sham mice. The WT-IR mice had the most pronounced IRI. Immunohistochemical staining revealed that more neutrophil and macrophage infiltration was seen in the left lung of WT-IR compared with the PAI-1 KO IR and WT-sham mice. These results indicate that the plasminogen activator/PAI-1 system may play an important role in recruitment of inflammatory cells during lung transplantation.

**Physiologic Data**

As shown in Figure 4, there was a significant protection in pulmonary compliance, pulmonary artery pressures, and airway resistance in the PAI-1 KO IR mice compared with the WT-IR mice. The mean pulmonary compliance in the WT-sham group was $5.587 \pm 0.547 \, \text{mL/cm H}_2\text{O}$; this was not statistically different from the mean compliance in the PAI-1 KO IR group, which was $4.292 \pm 0.377 \, \text{mL/cm H}_2\text{O}$ ($P = .08$). These values were markedly better than the mean compliance, $2.552 \pm 0.202 \, \mu\text{L/cm H}_2\text{O}$, in the WT-IR mice lungs ($P = .003$). The mean pulmonary artery pressures were markedly higher in the WT-IR mice, $12.53 \, \text{cm H}_2\text{O}$, compared with both PAI-1 KO IR ($9.23 \, \text{cm H}_2\text{O}$) and WT-sham mice ($5.70 \, \text{cm H}_2\text{O}$) ($P < .0001$). In terms of airway resistance, the WT-IR was also significant worse, $2.240 \pm 0.287 \, \text{cm H}_2\text{O}/\mu\text{L/s}$, compared with PAI-1 KO IR ($1.240 \pm 0.114 \, \text{cm H}_2\text{O}/\mu\text{L/s}$) and sham-IR (0.788 $\pm 0.038 \, \text{cm H}_2\text{O}/\mu\text{L/s}$). Unlike pulmonary compliance, in which there was no significant difference between PAI-1 KO IR and sham-IR, the protection compared with WT-IR was only partial. Pulmonary artery pressure and airway resistance with PAI-1 KO IR had higher pulmonary artery pressures and airway resistance compared with sham-IR ($P < .0001$ and $P = .004$, respectively). Taken together, these data show the IRI in PAI-1 KO IR mice was less when compared
with that in WT-IR mice, although there was still evidence of some injury. These data corroborate the histology and immunohistochemistry data.

**Chemokine/Cytokine Data**

TNF-α in plasma samples was significantly lower in the PAI-1 KO IR mice (0.136 ± 0.007 ng/mL) than in the WT-IR mice (0.270 ± 0.054 ng/mL; *P* = .027). MCP-1 in plasma samples was also significantly lower in the PAI-1 KO IR (0.026 ± 0.0002 ng/mL) than in WT-IR mice (0.039 ± 0.005 ng/mL; *P* = .017). MIP-2 trended lower in plasma samples in the PAI-1 KO IR mice than in the WT-IR mice, but this did not reach statistical significance (*P* = .190). However, in all the PAI-1 KO IR mice, MIP-2 levels were below detection limit of the ELISA. In the WT-IR mice, MIP-2 levels were detectable (0.604 ± 0.373 ng/mL) (see Figure 5, A). KC levels were below the detection limits in the plasma samples in both groups. In BAL samples, TNF-α, KC, and MIP-2 were significantly lower in the PAI-1 KO IR group (0.003 ± 0.0002 ng/mL, 0.007 ± 0.016 ng/mL, and 0.029 ± 0.004 ng/mL, respectively) than in the WT-IR group (0.005 ± 0.0006 ng/mL, 0.019 ± 0.002 ng/mL, and 0.078 ± 0.0197 ng/mL; respectively) (*P* = .003, .0006, and .027, respectively) (see Figure 5, B). MCP-1 levels in the BAL were below detection limits in both groups. Taken together, these data suggest that local abnormalities of fibrinolysis in IR affect the various inflammatory cytokines/chemokines locally, and these effects may also be reflected in the systemic circulation. We also performed ELISAs on whole lung homogenates as well as extracted total RNA from the left lung for real-time PCR, and there did not appear to be a significant different in any of the measured cytokines/chemokines (Figure 5, C) which suggests the intra-alveolar inflammatory component may be most affected locally.
DISCUSSION

A substantial body of evidence supports the idea that coagulation, fibrinolysis, and extravascular fibrin deposition play a role in acute lung injury and subsequent repair.\textsuperscript{6} Deposition of alveolar fibrin is characteristic of a wide range of acute insults in lung models. In the acutely injured lung, intravascular thrombosis can also occur. Extravascular fibrin deposition promotes an acute inflammatory response and lung dysfunction.\textsuperscript{6} Alveolar deposition is also characteristic of diffuse alveolar damage that occurs from various insults.\textsuperscript{6} Evidence supports transitional fibrin neomatrix involved in pathogenesis of acute inflammatory response in acute lung injury.

We have previously investigated the effects of hypoxia on the fibrinolytic system.\textsuperscript{5} Mice exposed to hypoxia were found to have an increase in plasma levels of PAI-1 and a decrease in tissue-type plasminogen activator and urokinase-type plasminogen activator. The co-localization studies with immunohistochemistry identified macrophages as the predominant source of increased PAI-1 within the hypoxic lung.\textsuperscript{7}

In our IRI model, we found decreased deposition of fibrin in the PAI-1 KO IR mice and a protective effect on injury in these mice. We do not know from these studies in which compartment(s) of the lung (intravascular, interstitium or intra-alveolar) the decrease in fibrin is seen, but it does appear that with this decrease in fibrin there is a decrease in inflammation. We have previously reported on the interplay between the fibrinolytic system and inflammation in IRI. After IR interleukin 10 (IL-10), null mice (IL-10 suppresses macrophage activation and downregulates proinflammatory cytokine production) were found to have exaggerated pulmonary PAI-1 expression compared with mice with IL-10. These mice null for IL-10 showed poor postischemic function and survival compared with mice with IL-10.\textsuperscript{13} Furthermore, in this study, recombinant IL-10, when given to mice null for IL-10 normalized the PAI/tissue-type plasminogen activator ratio, reduced pulmonary vascular fibrin deposition and rescued mice from lung injury.\textsuperscript{13}

Farivar and colleagues\textsuperscript{14} have also reported on the cross-talk between thrombosis and inflammation in a rat lung reperfusion injury model. In this article they used a specific direct thrombin inhibitor, hirudin, and showed that it protected against IRI. They also suggested mechanistically that the protective effects of thrombin inhibition were a result of decreased inflammatory pathway activation. Thrombin exerts proinflammatory influences on neutrophils, endothelial cells, and macrophages, and thus inhibition of thrombin with hirudin prevents this proinflammatory activation.

Our current findings are additive to our previous studies and those of Farivar and colleagues. The PAI-1 KO mice prevent fibrin accrual in the lung by increasing fibrinolysis. We saw less of an inflammatory response with less neutrophil infiltration, less cytokine and chemokine release, and improvement in physiologic parameters with the PAI-1 KO mice compared with controls.

The importance of PAI-1 in clinical lung transplantation is suggested by a study performed by Christie and colleagues.\textsuperscript{15} They found higher PAI-1 levels in plasma of human lung transplant recipients with severe IRI than in plasma of patients without IRI. This increase in plasma PAI-1 levels has also been seen in patients with adult respiratory distress syndrome compared with critically ill control patients.\textsuperscript{16}

The transitional fibrin that is found at the time of acute lung injury has been linked to various pulmonary fibrotic conditions.\textsuperscript{6} Not only does tissue fibrin influence inflammation, it also affects tissue repair responses. The presence and severity of IRI is a major risk factor in the development of chronic allograft rejection.\textsuperscript{3,4} Chronic allograft rejection in the lung is a fibro-obliterative process of the small airways. Although this study does not investigate the role played by fibrin deposition in the subsequent development of chronic allograft lung rejection, it does suggest that one way IRI may increase the risk of development of chronic rejection is via alterations in the fibrinolytic system.

The current study focuses on impaired fibrinolysis. The protection seen with the PAI-1 KO mice was only partial but significant in our studies. We plan future studies to look at whether increased coagulation also contributes to IRI. In human lung transplant recipients that have severe IRI, evidence of enhanced coagulation is seen by decreased levels of protein C.\textsuperscript{15}

In summary, our study provides evidence to support the hypotheses that PAI-1 plays an important role during lung IRI via (1) decreasing fibrin deposition, (2) suppressing cytokine/chemokines involved in inflammation and chemotaxis, and (3) blocking inflammatory cells (such as neutrophil and macrophage) infiltration into the injured lung tissues. Our data indicate that the association between fibrinolysis and inflammation in our lung IRI model may provide potential novel therapeutic targets for lung transplantation. For example, treatment with angiotensin-converting enzyme inhibitors quinapril (40 mg) and ramipril (10 mg) have been reported to decrease plasma PAI-1 antigen and PAI-1 activity significantly, but without affecting the level and activity of tissue plasminogen activator in patients.\textsuperscript{17,18} Therefore, angiotensin-converting enzyme inhibitors could be administered to the donor to enhance fibrinolysis when heparin is given already as a routine before cold flush perfusion to prevent thrombosis. We will further investigate whether this early stage protection will attenuate bronchiolitis obliterans development during late stages of lung transplantation using our heterotopic tracheal transplantation model.
References


