Lung protective mechanical ventilation

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Chapter 15

Plasminogen Activator Inhibitor type I Deficiency Attenuates Neutrophil Influx in Ventilator–induced Lung Injury in Mice

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Abstract

**Background:** Bronchoalveolar plasminogen activator inhibitor type–1 (PAI–1) levels increase with pulmonary inflammation. We determined the effects of mechanical ventilation in PAI–1 gene deficient mice in a model of ventilator–induced lung injury (VILI).

**Methods:** PAI–1 gene deficient mice and wild type mice were tracheotomized and mechanically ventilated for 5 hours with either lower tidal volumes (LV, ~7.5 ml/kg) or higher tidal volumes (HV, ~15 ml/kg). Spontaneously breathing mice served as controls. Endpoints of VILI were pulmonary coagulopathy (bronchoalveolar thrombin–antithrombin complex levels and D–dimer levels), lung wet to dry ratios (W/D), total protein level in bronchoalveolar lavage fluid (BALF), neutrophil influx, histopathology, and pulmonary and plasma cytokine levels (tumor necrosis factor–alpha, interleukin 6) and chemokine levels (macrophage inflammatory protein 2, keratinocyte–derived chemokine).

**Results:** Mechanical ventilation resulted in lung injury, with more pulmonary coagulopathy, higher total protein in BALF and more neutrophil influx in both ventilated groups. W/D was only higher in HV–mice as compared to control mice. Higher cytokine levels were found in the pulmonary compartment, with higher levels in the HV–group. In PAI–1 gene deficient mice the influx of neutrophils in the pulmonary compartment was attenuated, but increased levels of pulmonary cytokines and chemokines were found with VILI. Other endpoints of VILI were not different between PAI–1 gene deficient mice and wild type mice.

**Conclusions:** PAI–1 gene deficiency attenuates recruitment of neutrophil cells during mechanical ventilation.
**Introduction**

Pulmonary coagulopathy is a characteristic feature of pneumonia and acute lung injury (ALI), or its more severe form the acute respiratory distress syndrome (ARDS) [1,2]. Disturbed fibrin turnover with pulmonary inflammation results from localized tissue factor–mediated thrombin generation, impaired activity of natural coagulation inhibitors and depression of bronchoalveolar urokinase plasminogen activator–mediated fibrinolysis. The impaired fibrinolysis is caused by an increase of plasminogen activator inhibitors [3-5]. Indeed, in patients with pneumonia–related coagulopathy elevated plasminogen activator inhibitor–1 (PAI–1) levels in bronchoalveolar lavage fluid have been found [1,3,5,6]. Elevated PAI–1 levels in patients with pneumonia correlate with poor outcome [6,7]. Similarly, in ALI/ARDS elevated plasma levels of PAI–1 are associated with increased morbidity and mortality [8].

Recently, in a rat model of pulmonary coagulopathy we demonstrated mechanical ventilation (MV) with high tidal volumes (VT), mimicking injurious MV resulting in ventilator–induced lung injury (VILI), to attenuate the fibrinolytic activity in the lungs [9]. This appeared to be caused, at least in part, by increased local production of PAI–1. Similar findings were encountered in an endotoxin–induced lung injury model in rats [10]. In addition, we recently showed short–term MV with injurious settings (high VT and no positive end–expiratory pressure [PEEP]) in patients without preexisting lung injury to cause upregulation of PAI–1 in the pulmonary compartment [11]. Lung–protective MV–settings (low VT and PEEP) attenuated this rise.

The aim of the present investigation was to determine more exactly the role of PAI–1 in the pathogenesis of pulmonary coagulopathy with MV. In addition, we determined whether attenuation of pulmonary fibrin production alters the local inflammatory response. To this end we used a VILI–model in mice using lower VT and higher VT.

**Materials and Methods**

The study was approved by the Animal Care And Use Committee of the Academic Medical Center of the University of Amsterdam, Amsterdam, the Netherlands. Animal procedures were carried out in compliance with Institutional Standards for Human Care and Use of Laboratory Animals.

**Mice**

Normal C57Bl/6 wild type (WT) mice (n = 36) were obtained from Harlan Sprague–Dawley (Horst, the Netherlands). PAI–1 gene deficient (PAI-1/-) mice on a C57Bl/6 genetic background (n = 36) were obtained from the Jackson Laboratory (Bar Harbor, ME). PAI-1/- exhibit normal fertility, viability, tissue histology and development, and show no evidence of hemorrhage [12]. Females with weights ranging from 18 – 22 grams were used in all
experiments. Non–ventilated mice served as controls (n = 12 for either WT or PAI-1/-mice). Intubated and mechanically ventilated mice were ventilated with 2 different MV–strategies (see below). In total this results in 6 groups of animals (see below).

Instrumentation and anesthesia

Throughout the experiments rectal temperature was monitored and maintained between 36.0 – 37.5°C using a warming path. Anesthesia was achieved with intraperitoneal injection of a mix of ketamine (Eurovet Animal Health B.V., Bladel, the Netherlands), medetomidine (Pfizer Animal Health B.V., Capelle a/d IJssel, the Netherlands), and atropine (Pharmachemie, Haarlem, the Netherlands) (KMA). Induction anesthesia consisted of injection of KMA “induction”–mix: 7.5 µl per gram of body weight of 1.26 ml 100 mg/ml ketamine, 0.2 ml 1 mg/ml medetomidine, and 1 ml 0.5 mg/ml atropine in 5 ml normal saline. Maintenance anesthesia consisted of injection of 10 µl per gram body weight of KMA “maintenance”–mix, consisting of 0.72 ml 100 mg/ml ketamine, 0.08 ml 1 mg/ml medetomidine, and 0.3 ml 0.5 mg/ml atropine, in 20 ml normal saline. Maintenance–mix was administered via an intraperitoneal catheter (PE 10 tubing, BD, Breda, the Netherlands) every hour.

Mechanical ventilation strategies

A Y–tube connector, 1.0 mm outer diameter and 0.6 mm inner diameter (VBM Medizintechnik GmbH, Sulz am Neckar, Germany) was surgically inserted into the trachea under general anesthesia. Mice were placed in a supine position and connected to a human ventilator (Servo 900 C, Siemens, Solna, Sweden). Mice were pressure controlled ventilated with either an inspiratory pressure of 10 cmH2O (resulting in lung–protective VT ~ 7.5 ml/kg; low VT, LV1) or an inspiratory pressure of 18 cmH2O (resulting in injurious VT ~ 15 ml/kg; high VT, HV1). Respiratory rate was set at 120 breaths/min and 70 breaths/min with LV1 and HV1, respectively. These respiratory settings resulted in normal PaCO2 values after 5 h of MV. PEEP was set at 2 cmH2O with both MV–strategies. The fraction of inspired oxygen was kept at 0.5. The inspiration to expiration ratio was kept at 1:1 throughout the experiment. A sigh (sustained inflation with 30 cmH2O) for 5 breaths was performed every 30 minutes. Mice received an intraperitoneal bolus of 1 ml normal saline 1 hour before start of anesthesia and initiation of MV, followed by 0.2 ml sodium bicarbonate (200 mmol/l NaHCO3) administered via the intraperitoneal catheter every 30 minutes until the end of MV.

Hemodynamic and ventilatory monitoring

Systolic blood pressure and heart rate were non–invasively monitored using a murine tail–cuff system (ADInstruments, Spenbach, Germany). Blood pressure and pulse were measured directly after start of MV, after 2.5 and 5 hour of MV. The data were recorded
on a data acquisition system (PowerLab/4SP, ADInstruments). Systolic blood pressure and heart rate were averaged from three consecutive measurements.

$V_T$ was checked hourly with a plethysmograph system. A minimum of 5 consecutive breaths was selected for analysis of the digitized $V_T$–signals.

**Study groups**

Non–ventilated control mice (WT and PAI-1/- mice) received half the dose of induction anesthesia, were spontaneously breathing and sacrificed after 5 hours.

LV$T$–mice and HV$T$–mice (WT and PAI-1/- mice) were mechanically ventilated for 5 hours and then sacrificed. Half of these mice were sacrificed and blood was drawn from the vena cava inferior into a sterile syringe, transferred to EDTA–coated tubes and immediately placed on ice. Plasma of two mice was pooled together. Subsequently, bronchoalveolar lavage fluid (BALF) was obtained from the right lung; the left lung was used to measure wet to dry ratios. The other half of these mice were used for blood gas analysis studies. Blood was sampled from the carotid artery. The lungs of these mice were used for homogenate (right lung) and histopathology (left lung).

**Blood gas analysis**

For blood gas analysis, blood was immediately analyzed in a Rapidlab 865 blood gas analyzer (Bayer, Mijdrecht, the Netherlands). The other blood samples were centrifuged at 3000 rpm at 4°C for 10 min and the supernatants were aliquoted and frozen at -20°C until assayed.

**Lung wet to dry ratios (W/D)**

For W/D the left lung was weighed and subsequently dried for three days in an oven at 65°C. The ratio of wet weight to dry weight represents tissue edema.

**Lung homogenates**

During sacrificing the right lung was removed and snap frozen in liquid nitrogen. These frozen specimens were suspended in 4 volumes of sterile isotonic saline and subsequently lysed in 1 volume of lysis buffer (150 mM NaCl, 15 mM Tris [tris(hydroxymethyl)aminomethane], 1 mM MgCl$_2$, 1mM CaCl$_2$, 1% Triton X-100, 100 μg/ml pepstatin A, leupeptin and aprotinin, pH 7.4) and incubated at 4°C for 30 min. Homogenates were spun at 3400 rpm at 4°C for 15 minutes after which the supernatants were stored at −20°C until assayed.

**Bronchoalveolar lavage**

Bronchoalveolar lavage fluid (BALF) was obtained by instilling three times 0.5 ml aliquots of saline by a 22–gauge Abbocath–T catheter (Abbott, Sligo, Ireland) into the trachea.
Approximately, 1.1 ml of lavage fluid was retrieved per mouse and cell counts were determined using a hemacytometer (Beckman Coulter, Fullerton, CA). Subsequently, differential counts were done on citospin preparations stained with a modified Giemsa stain, Diff–Quick (Dade Behring AG, Düdingen, Switzerland). Supernatant was stored at -80° C for total protein level and thrombin–antithrombin complexes.

Histopathology
For histopathology lungs were fixed in 4% formalin and embedded in paraffin. 4μm sections were stained with hematoxylin–eosin (H&E), and analyzed by a pathologist who was blinded for group identity. To score lung injury we used a modified VILI histology scoring system as previously described [13]. In short, four pathologic parameters were scored on a scale of 0 – 4: (a) alveolar congestion, (b) hemorrhage, (c) leukocyte infiltration, and (d) thickness of alveolar wall/hyaline membranes. A score of 0 represents normal lungs; 1, mild, < 25% lung involvement; 2, moderate, 25 – 50% lung involvement; 3, severe, 50 – 75% lung involvement and 4, very severe, > 75% lung involvement. The total histology score was expressed as the sum of the score for all parameters.

Assays
Total protein levels in BALF were determined using a Bradford Protein Assay Kit (OZ Biosciences, Marseille, France) according to manufacturers’ instructions with bovine serum albumin as standard. Cytokine and chemokine levels in lung homogenates were measured by enzyme–linked immunosorbent assay (ELISA) according to the manufacturer’s instructions. Tumor necrosis factor–alpha (TNF), interleukin (IL)–6, macrophage inflammatory protein (MIP)–2 and keratinocyte–derived chemokine (KC) assays were all obtained from R&D Systems (Abingdon, UK). Thrombin–antithrombin complex levels in BALF were measured with a mouse specific ELISA as previously described [14]. To measure plasmin activity, concentrations of D–dimer, a split product cleaved off from cross–linked fibrin by a direct action of plasmin, were measured. D–dimer was measured with ELISA (Asserachrom D–dimer, Roche, Woerden, the Netherlands).

Statistical analysis
All data in the results are expressed as means ± SD or median ± interquartile range [IQR], where appropriate. To detect differences between mechanical ventilation groups, the Dunnett method, in conjunction with two–way analysis of variance, was used. For differences between PAI–1/– mice and WT mice, post–hoc analysis with Mann Whitney U test was done. A p value of < .05 was considered significantly. All statistical analyses were carried out using SPSS 12.0.2 (SPSS, Chicago, IL).
Results

*Hemodynamic and ventilator monitoring*

All instrumented animals survived 5 hours of MV after which they were sacrificed. Control animals survived anesthesia and were also sacrificed after 5 hours. Hemodynamic monitoring demonstrated stable conditions throughout the experiment (figure 1): systolic blood pressure and heart rate remained stable in all animals for the complete duration of MV, with no differences between PAI-1/- mice and WT mice. Blood gas analysis showed no differences between PAI-1/- mice and WT mice (data not shown).

**Figure 1** Hemodynamic parameters during 5 hours of mechanical ventilation: Systolic blood pressure (A) and heart rate (B; beats/min) was measured at three time points (t = 0, 2.5 and 5 hours after start of mechanical ventilation) in mice ventilated with low tidal volumes (Low VT) and high tidal volumes (High VT). Each mentioned group consisted of plasminogen activator inhibitor 1 knockout (PAI-1/-) mice and WT mice. Data represent means of 12 mice.

*Pulmonary coagulopathy*

BALF TATc–levels were higher in both ventilation groups as compared to control mice (p = .041 for LVt–mice and p < .001 for HVt–mice), with higher levels in HVt–mice (p < .001 vs. low VT–mice; figure 2A). In accordance, D–dimer–levels were higher in both ventilated groups as compared to control (p < .001 for LVt–mice and HVt–mice), also with higher levels in HVt–mice (p < .001 vs. low VT–mice; figure 2B).

*Lung injury*

Lung W/D was higher in HVt–mice as compared to LVt–mice (p < .001) and controls (p < .001; figure 3A). In accordance, total BALF protein levels were higher in both ventilation groups (p = .002 for LVt and p < .001 for HVt mice), with higher levels in HVt–mice (p <
.001; figure 3B). The histopathological changes were minor. None of the lungs showed signs of inflammation, congestion or hyaline membranes. Only hemorrhage could be seen, which was not different among both ventilation groups.

**Figure 2** Thrombin-antithrombin complexes (TATc) levels in bronchoalveolar lavage fluid (A) and D-dimer levels in long homogenate (B) after 5 hours in control (C) mice, mice ventilated with low tidal volumes (LV) and high tidal volumes (HV). Each mentioned group consisted of plasminogen activator inhibitor (PAI) 1 knockout (PAI-1/-) mice and WT mice. Data represent median (IQR) of six mice. *p < .05 vs. WT; †p < .01 vs. WT.

**Pulmonary and systemic inflammation**

Ventilated mice demonstrated higher pulmonary levels of TNF and IL–6 as compared to control mice (p ≤ .001 for LV and HV mice). For both cytokines there was no significant difference between LV and HV mice, however (Figure 4A and 4B). Ventilated mice also demonstrated higher pulmonary levels of MIP–2 and KC as compared to control (p = .002 for LV mice and p < .001 for HV mice), with higher levels of MIP–2 and KC in HV mice (p = .001 vs. LV mice; Figure 4C and 4D). The number of neutrophils in BALF was higher in HV mice as compared to control (p < .001), and a trend for more neutrophils in LV mice as compared to control (p = 0.094). The difference between HV and LV mice was significant (p < .001; figure 3C).

Systemic levels of IL–6 and KC were elevated in both ventilation groups as compared to control (p ≤ .002 for both LV and HV mice), with higher levels in HV mice (p = .046 for IL-6 and p = .005 for KC; figure 5).
Effect of MV in PAI–1/– mice

There was no significant difference between PAI–1/– mice and WT mice regarding activation of coagulation in the pulmonary compartment (figure 2A). In PAI-1/– mice fibrinolysis was enhanced during MV, as reflected by a stronger increase in D–dimer levels (figure 2B).

Also, no differences were seen between PAI-1/– mice and WT–mice regarding W/D and total protein in BALF (figure 3A and 3B). Histopathological changes (only hemorrhage) were not different between PAI-1/– mice and WT mice.

In PAI–1/– mice there was less neutrophil influx in both ventilated groups as compared to WT mice (figure 2C). PAI–1/– mice demonstrated higher levels of IL–6 in non–ventilated control mice (p = .028). There was trend for higher IL–6 levels in HV–mice as compared to WT mice (p = .059). PAI–1/– mice also demonstrated higher pulmonary levels of TNF in all groups (control, LV/– and HV/–mice; figure 4A and 4B). PAI–1/– mice demonstrated higher levels of MIP–2 and KC. For MIP–2 pulmonary levels in PAI–1/– mice were significantly higher in control mice and LV/–mice as compared to WT mice (p = .003 for control mice and p = .008 for LV/–mice). PAI–1/– mice also demonstrated higher pulmonary levels of KC in HV/–mice (p = .012; figure 4C and 4D).

Plasma levels of IL–6 and KC were not different between WT and PAI–1/– mice (figure 5).

Discussion

In this model of VILI we found more lung injury, more pulmonary coagulopathy, higher W/D and higher total protein in BALF in mice ventilated with HV/–. There was also more pulmonary inflammation, as reflected by higher cytokine and chemokine levels and more neutrophil influx in the pulmonary compartment, in mechanically ventilated mice. Of note,
PAI–1/- mice showed less neutrophil influx in the pulmonary compartment, together with locally increased levels of pulmonary cytokines and chemokines. However, no differences were seen regarding other endpoints of VILI between PAI–1/- mice and WT mice.

Our results are in line with several other reports. First, in a *Klebsiella pneumoniae* pneumonia model in mice, PAI-1/- mice demonstrated less infiltrating neutrophils in their lungs [15]. In this study, higher pulmonary IL–6 levels in PAI-1/- mice were found, and higher KC levels in plasma. A diminished influx of neutrophils into the BALF of PAI-1/- mice has also been found after aerosol LPS exposure, which was associated with elevated intravascular KC levels [16]. In our model of MV, pulmonary levels of KC in HV, PAI-1/- mice were higher as compared to WT. However, plasma levels of KC were comparable and there was no chemotactic gradient. Our findings are also in line with the results obtained in a model of bleomycin–induced lung injury, in which PAI–1 gene deficiency protected...
against inflammation–induced lung damage and over–expression of PAI–1 enhanced the accumulation of neutrophils in the lung [17]. Furthermore, in an antigen–induced arthritis model PAI–1/– mice showed significantly reduced joint inflammation [18]. Finally, in a model of glomerulonephritis PAI–1 deficiency reduced the number of infiltrating neutrophils in the glomeruli, while mice over–expressing PAI–1 showed a profound increase in neutrophil infiltration [19]. Together, these data strongly suggest that the role of PAI–1 in inflammatory cell migration is often stimulatory.

**Figure 5** Systemic levels of interleukin (IL)-6 (A) and keratinocyte-derived chemokine (KC) (B) in plasma after 5 hours in control (C) mice, mice ventilated with low tidal volumes (LV$_T$) and high tidal volumes (HV$_T$). Each mentioned group consisted of plasminogen activator inhibitor 1 knockout (PAI-1/–) mice and WT mice. Data represent mean (SEM) of six mice. The levels of IL-6 and KC in control mice were below the detection limit of the assay.

Neutrophil recruitment to the lung is believed to be a critical step in the pathogenesis of ALI/ARDS, and results from the release of a milieu of cytokines and chemokines that precedes ALI/ARDS [20,21]. Recently, it was shown that a neutrophil elastase inhibitor could attenuate VILI in mice. Indeed, mice that received the neutrophil elastase inhibitor showed complete inhibition of neutrophil elastase and myeloperoxidase activities, attenuation of neutrophil accumulation and lung water content [22]. Our data extend these findings by showing that neutrophil infiltration is attenuated in PAI–1/– mice in a model of VILI.

VILI was clearly present with the use of HV$_T$ after 5 hours of MV. Interestingly, with LV$_T$ VILI also developed, although to a lesser extent. This finding is in accordance with a previous report, in which it was reported low V$_T$ (8 ml/kg) for 4 hours to result in a reversible inflammatory reaction, while preserving tissue integrity [23]. Thus, even the use of LV$_T$ may be considered injurious when ventilating mice. Of note, however, this finding is not supported by human data. Patients ventilated with lower V$_T$ (6 ml/kg) for 5 hours did not
show upregulation of pulmonary inflammatory mediators and markers of coagulation as opposed to patients ventilated with conventional $V_T$ (12 ml/kg) [11,24]. Unrecognized differences in MV between mice and the human setting may be responsible for this difference.

Our model has several limitations. LV$_T$ in the present study are equal to that of spontaneously breathing C57Bl/6 mice (6 – 9 ml/kg) [25]. However, MV with LV$_T$ may promote development of atelectasis. In our model we used deep inflation every 30 minutes to prevent atelectasis. Periodic deep inflation can open the lung, but if delivered too frequently may cause damage via repeated overdistension by itself [26]. It may be speculated that the lungs were not recruited frequently enough to prevent atelectasis, and indeed some pulmonary inflammation seen in LV$_T$–mice may be caused by atelectotrauma. Alternatively, even MV using a $V_T$ of 7.5 ml/kg may still cause overdistension of alveoli. Second, $V_T$ in HV$_T$–mice are still quite large. Although there is still underuse of lung–protective ventilation with the use of lower $V_T$ [27,28], $V_T$ have declined gradually over the past 10 years [29,30]. However, $V_T$ of as large as 15 ml/kg are reported to be used in human clinical studies [31,32]. Therefore our comparison may still reveals relevant information on lung injury caused by MV.

In conclusion, PAI–1 gene deficiency attenuates recruitment of neutrophils into the alveolar space during mechanical ventilation. This suggests PAI–1 to play a stimulatory role of cell migration into the alveoli, which is independent of local production of chemokines. Further studies are needed before inhibition of PAI–1 is to be tested in clinical trials of patients with or at risk for ventilator–associated lung injury.
References