Neutrophil Elastase Is Needed for Neutrophil Emigration into Lungs in Ventilator-Induced Lung Injury

A. Murat Kaynar¹, A. McGarry Houghton², Esther H. Lum³, Bruce R. Pitt⁴, and Steven D. Shapiro¹

¹Department of Critical Care Medicine, ²Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania; ³Washington University School of Medicine, St. Louis, Missouri; and ⁴Department of Environmental and Occupational Health, University of Pittsburgh, Graduate School Public Health, Pittsburgh, Pennsylvania

Mechanical ventilation, often required to maintain normal gas exchange in critically ill patients, may itself cause lung injury. Lung-protective ventilatory strategies with low tidal volume have been a major success in the management of acute respiratory distress syndrome (ARDS). Volutrauma causes mechanical injury and induces an acute inflammatory response. Our objective was to determine whether neutrophil elastase (NE), a potent proteolytic enzyme in neutrophils, would contribute to ventilator-induced lung injury. NE-deficient (NE^{-/-}) and wild-type mice were mechanically ventilated at set tidal volumes (10, 20, and 30 ml/kg) with 0 cm H₂O of positive end-expiratory pressure for 3 hours. Lung physiology and markers of lung injury were measured. Neutrophils from wild-type and NE^{-/-} mice were also used for *in vitro* studies of neutrophil migration, intercellular adhesion molecule (ICAM)-1 cleavage, and endothelial cell injury. Surprisingly, in the absence of NE, mice were not protected, but developed worse ventilator-induced lung injury despite having lower numbers of neutrophils in alveolar spaces. The possible explanation for this finding is that NE cleaves ICAM-1, allowing neutrophils to egress from the endothelium. In the absence of NE, impaired neutrophil egression and prolonged contact between neutrophils and endothelial cells leads to tissue injury and increased permeability. NE is required for neutrophil egression from the vasculature into the alveolar space, and interfering with this process leads to neutrophil-related endothelial cell injury.

Keywords: neutrophil elastase; ventilator-induced lung injury; endothelial injury; emigration

Mechanical ventilation provides effective gas exchange in patients with respiratory failure. Since its inception, questions about the possibility of mechanical ventilation causing lung injury have been raised, giving rise to the term “ventilator-induced lung injury” (VILI) (1). A growing body of literature documented harmful effects of alveolar overdistention during mechanical ventilation (2). In animal models, increasing tidal volumes cause an acute inflammatory response with neutrophil infiltration and disruption of the endothelial and epithelial barriers (1, 3).

Pre-existing lung injury may further exacerbate inflammation and VILI (4). As most patients requiring mechanical ventilation have a primary pulmonary insult, distinguishing mechanical ventilator-induced injury from an underlying disease process has been difficult. Moreover, with heterogeneous

CLINICAL RELEVANCE

This study suggests a role for neutrophil elastase in neutrophil emigration out of vasculature during mechanical ventilation. The lack of the enzyme caused prolonged interaction between neutrophils and endothelium leading to endothelial injury.

injury to the lung, the relatively spared, disease-free lung tissue, may be particularly sensitive to overdistention (5). Based upon these concerns, strategies using low tidal volume ventilation have been adopted, and resulted in improvement in mortality in acute respiratory distress syndrome (ARDS) (6).

How pure mechanical ventilation leads to neutrophil sequestration into the lung tissue with subsequent injury in VILI is not well understood. Raised alveolar and transpulmonary pressures may produce temporary neutrophil entrapment due to compression of alveolar capillaries (7). Neutrophil recruitment can be sustained by induction of local inflammatory mediators (3) such as CXC chemokines (KC and MIP-2) that have been shown in murine models of VILI to cause neutrophil and microvascular endothelial cell stiffening, activation, migration, and endothelial cell discontinuity with resultant edema (7–9).

In the systemic circulation, neutrophils egress from vasculature through post-capillary venules, whereas this event occurs in alveolar capillaries in the lung (10). Within the pulmonary capillaries, activated neutrophils become stiff and trapped and then tightly adhere to capillary endothelium. These neutrophils migrate directly through pulmonary capillaries through a process that is only partially β_2 -integrin-dependent (11). Neutrophils subsequently detach from high-affinity binding sites, alter their cytoskeleton, and open tight junctions between endothelial cells, allowing them to egress from the vasculature (12). The role of proteinases in neutrophil migration and lung injury, particularly neutrophil elastase (NE), has been a major focus of investigation for years. Because of its destructive potential, NE has been thought to cause or amplify acute and chronic lung injury including ARDS (13). With respect to neutrophil migration, most cell-based studies failed to support the hypothesis that NE promotes neutrophil migration through proteolytic degradation of extracellular matrix components (14), but NE might participate through other mechanisms. We and others have previously generated NE-null mutant mice (NE^{-/-}) to study the functional properties of NE. Our main finding was that NE was required for neutrophil antimicrobial activity. Otherwise, neutrophil development and function was unaltered (15, 16). NE^{-/-} mice have not been used to directly assess neutrophil migration through endothelial barriers.

The purpose of this study was to investigate the role of NE in the earliest phases of VILI. In contrast to our initial hypothesis that mice lacking NE would be protected from VILI, we found that lack of NE promoted lung injury due to prolonged endothelial-neutrophil interaction in the absence of NE. Further studies demonstrated the role for NE in mediating trans-

(Received in original form April 25, 2007 and in final form January 25, 2008)

This work was supported by grants FAER-RTG (A.M.K.), National Institutes of Health HL65697 (B.R.P.), and NIH HL054853-11A1 (S.D.S.).

Correspondence and requests for reprints should be addressed to A. Murat Kaynar, MD, Department of Critical Care Medicine, Scaife Hall 639, University of Pittsburgh School of Medicine, 3550 Terrace Street, Pittsburgh, PA 15261. E-mail: kaynarm@upmc.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 39, pp 53–60, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-03150C on February 14, 2008

Internet address: www.atsjournals.org

vascular egress via cleavage of intercellular adhesion molecule (ICAM)-1.

MATERIALS AND METHODS

Additional methodological details are provided in the online supplement.

Mice

Mice deficient in NE (NE^{-/-}) were generated in our laboratory by gene targeting (15) and backcrossed 10 generations into the C57BL/6J background. Of note, deletion of NE did not alter expression of other proteinases and did not alter neutrophil development.

Complete Blood Counts

Complete blood and leukocyte differential counts were determined in NE^{-/-} and wild-type (WT) mice using a Baker Instruments 9000 Hematologic Series cell counter (BioImmuno Chem, Allentown, PA) after cytocentrifugation and staining with Diff Quick Stain (Harleco, Gibbstown, NY).

Animal Preparation, Experimental Ventilation Protocols, and Physiological Measurements

Mice were ventilated using a small animal ventilator (flexiVent, SCIREQ, Montreal, Quebec, Canada) via tracheostomy, and a polyethylene catheter was introduced into right carotid artery for monitoring arterial pressures and infusion of fluids. We created VILI by applying various tidal volumes—10, 20, or 30 ml/kg—during a 3-hour experimental period with 0 cm H₂O of positive end-expiratory pressure (PEEP). Quasi-static pressure–volume (P–V) curves were generated every 30 minutes. Static compliance was calculated from the slope of each curve.

Bronchoalveolar Lavage

Total bronchoalveolar lavage (BAL) cell and differential leukocyte counts were performed at the end of experiments using PBS in 0.8 ml volume with five replicates.

Histopathological Examination of the Lungs

Lung tissues fixed with zinc fixative were sectioned and stained with Gr1 antibody for neutrophils. Total lung field, alveolar, and intravascular neutrophils were quantified. The intra-alveolar/intravascular ratio has previously been used to measure the extravasability of neutrophils into lung tissue (17).

Measurement of Wet:Dry Lung Weights

At the end of experiments, wet weight of the lungs were measured and then incubated in a 72°C oven for 24 hours. Wet:dry ratio of the left lung was measured as a surrogate marker of lung injury.

Neutrophil Isolation, Chemotaxis, and Matrigel Invasion Assays

Neutrophils from NE^{-/-} mice and WT littermates were collected after intraperitoneal injection of thioglycollate with greater than 90% purity. Neutrophils were used either in micro-chemotaxis or Matrigel invasion chambers to assess their chemotactic ability toward formyl-Met-Leu-Phe (fMLP), leukotriene B₄ (LTB₄), zymosan-activated serum (ZAS), or culture media as a negative control.

Transmigration of Neutrophils across Human Pulmonary Vein and Microvascular Endothelial Cell-Coated Membranes

To assess the ability of neutrophils to migrate across pulmonary endothelium, we seeded Transwell inserts with human pulmonary vein

endothelial cells (hPVEC) or human pulmonary microvascular endothelial cells (hPMVEC), and they were grown to confluence. Neutrophils (1×10^6) were allowed to migrate toward fMLP (10^{-5} M) or ZAS.

Endothelial Cell Injury after Co-Incubation and Transmigration of Neutrophils across hPVEC-Coated Membranes

To assess whether neutrophils in prolonged contact with endothelial cells would cause cell injury upon transmigration, we exposed ⁵¹Cr-labeled hPVEC grown to confluence on Transwell inserts to activated neutrophils. Neutrophils were allowed to co-incubate with endothelial cells for 18 hours. In additional experiments, neutrophils were allowed to migrate to the lower chamber in response to fMLP (10^{-5} M). Release of ⁵¹Cr was used as marker for endothelial cell injury.

ICAM-1 Expression on hPVEC after Co-Incubation with Neutrophils

To assess whether the increased NE^{-/-} neutrophil adhesion to endothelial cells is mediated by their impaired ability to cleave ICAM-1, we co-incubated PMA-activated WT and NE^{-/-} neutrophils with TNF- α -activated endothelial cells (ratio 10:1) at 37°C for 2 hours and cells were imaged using an inverted microscope. The cells were then either lysed and subjected to Western blot analysis for ICAM-1, or cells were fixed and stained for cell surface ICAM-1 expression by immunofluorescence.

Data Analysis

Data are expressed as means $\pm$ SD. Pairwise comparisons were made by Student's *t* tests. A *P* value of < 0.05 was considered significant. The values of the P–V loop areas were compared by use of paired two-tailed *t* tests.

RESULTS

Complete Blood Counts

Previous reports have linked mutations in the NE gene to the syndrome of cyclic neutropenia in humans (18). To assess potential effects of NE deficiency on circulating neutrophils, we measured WBC and neutrophil counts over a period of 22 days, the described cycle length for the recurrence of neutropenia. We found no difference in WBC and neutrophil counts obtained on Days 1, 3, 8, 10, 12, 15, 17, and 22 between WT and NE^{-/-} mice (Table 1).

Total Cell and Neutrophil Content in BAL after Mechanical Ventilation

To study the role of NE in the migration of inflammatory cells into the alveolar spaces, we measured the number of total cells and neutrophils in the BAL in WT and NE^{-/-} mice. We found that the numbers of total cells in BAL increased with increasing tidal volumes but were similar in WT and NE^{-/-} mice for similar ventilation groups (see online supplement). The difference between the two genotypes was not statistically significant. Neutrophils represent a small but important subset of inflammatory cells. Total neutrophil count in BAL also increased proportionally to *T_v* in the WT mice, but this increase in the alveolar neutrophil counts was significantly blunted in NE^{-/-} mice ($P < 0.05$) (Figure 1).

Static Lung Compliance after Mechanical Ventilation in WT and NE^{-/-} Mice

Static compliance, which was used as a marker of lung injury severity, was measured every 30 minutes throughout the 3-hour

TABLE 1. C57BL6 NE^{-/-} MICE HAVE NORMAL NUMBERS OF CIRCULATING NEUTROPHILS

	Day 1	Day 3	Day 5	Day 8	Day 10	Day 12	Day 15	Day 17	Day 22
WT (% neutrophil)	19.7 $\pm$ 7.2	26.5 $\pm$ 5.7	20.2 $\pm$ 6.2	23.5 $\pm$ 9.1	19.8 $\pm$ 6.7	22.2 $\pm$ 8.1	19.1 $\pm$ 7.5	20.9 $\pm$ 5.5	30 $\pm$ 10.1
NE ^{-/-} (% neutrophil)	21.1 $\pm$ 5.5	20.4 $\pm$ 8.8	19.3 $\pm$ 4.6	18.4 $\pm$ 3.3	21.9 $\pm$ 7.8	20.2 $\pm$ 7.8	23.1 $\pm$ 7.1	19.1 $\pm$ 8.9	24.1 $\pm$ 5.4

Definition of abbreviations: NE^{-/-}, mice deficient in neutrophil elastase; WT, wild-type mice.

Data presented are means $\pm$ SD (n = 10/group). No statistically significant differences were found between two groups.

experimental period after recruitment maneuvers to normalize physiologic properties. There was no difference in baseline lung compliance between the two genotypes, nor were there differences between the groups at 10 ml/kg Tv (WT: 0.068 and NE^{-/-}: 0.079 ml/cm H₂O). As expected, with increasing tidal volumes, lung functions worsened over time. In the 20 and 30 ml/kg Tv groups, compliance decreased over the course of the experimental period in both WT and NE^{-/-} mice as compared with 10 ml/kg group ($P < 0.05$) (Figure 2). Unexpectedly, the static lung compliance of NE^{-/-} mice was significantly worse as compared with sex- and age-matched control WT mice under similar ventilation conditions ($P < 0.05$).

Lung Injury as Measured by Wet:Dry Lung Weights after Mechanical Ventilation

Wet:dry lung weight ratios of WT and NE^{-/-} mice ($n = 5$) after 3-hour ventilation at varying tidal volumes were measured (Figure 3). In the 20 and 30 ml/kg Tv groups, both genotypes had significantly higher wet:dry ratios as compared with 10 ml/kg groups in their respective genotypes. At 20 ml/kg and 30 ml/kg Tv, NE^{-/-} mice had significantly higher extravascular lung water, consistent with increased lung stiffness above and suggestive of worse lung injury and edema in NE^{-/-} than WT control mice ($P < 0.05$).

Neutrophil Emigration from the Pulmonary Vasculature into the Alveolar Space after Mechanical Ventilation

We examined Gr1 immunostained lung sections under light microscopy to determine the ratio of neutrophils located in the airspace as opposed to those located in the intravascular space (Figure 4A). In WT mice following mechanical ventilation with 20 and 30 ml/kg Tv, the ratio of intra-alveolar/intravascular neutrophils was significantly higher in WT than NE^{-/-} mice in both Tv groups ($P < 0.05$), suggesting impaired ability of NE^{-/-} neutrophils to egress from the vasculature into the alveolar space. Representative images at 20 ml/kg tidal volume are shown (Figure 4B).

Chemotaxis of WT and NE^{-/-} Neutrophils

To determine the basis for the defect in neutrophil recruitment in NE^{-/-} mice, we first assessed neutrophil chemotaxis. Neutrophils from WT and NE^{-/-} mice were placed into a micro-chemotaxis

Figure 1. Neutrophil elastase (NE) is required for migration of neutrophils to alveolar space after mechanical ventilation in mice. Numbers of neutrophils entering the alveolar space were quantified by bronchoalveolar lavage in wild-type (WT) (solid bars) and NE^{-/-} (shaded bars) mice ($n = 6$ /group) after 3-hour mechanical ventilation at varying tidal volumes. Data presented are means $\pm$ SD. Statistically significant differences ($*P < 0.05$) were found between two groups at each ventilatory setting (10 ml/kg: 9,987 versus 776; 20 ml/kg: 31,000 versus 4,841; 30 ml/kg: 39,276 versus 5,886).

Figure 2. Decrease in static lung compliance with mechanical ventilation over time and with increasing lung volumes is worse in NE^{-/-} mice (shaded circles). Solid circles, WT mice. Static compliance (ml/cm H₂O) of closed chest animals was measured throughout the experimental period (3 h) every 30 minutes after recruitment maneuvers to normalize the physiologic properties. Time courses were obtained for (top panel) 10 ml/kg tidal volume, (middle panel) 20 ml/kg tidal volume, and (bottom panel) 30 ml/kg tidal volume. Data are presented are means $\pm$ SD. Statistically significant differences ($*P < 0.05$) were found between two groups at 20 and 30 ml/kg tidal volumes after 2 hours of mechanical ventilation.

(Boyden) chamber and the cells were incubated in the presence of agents known to be chemotactic for neutrophils: LTB₄, ZAS, and fMLP. WT and NE^{-/-} neutrophils displayed equal movement in response to the various chemotactic stimuli (Figure 5A). Hence, impaired transvascular migration in NE^{-/-} mice was not due to impaired motility or processing of chemokines.

Migration of WT and NE^{-/-} Neutrophils through Matrigel

Previous studies have used NE inhibitors to assess the role of NE in neutrophil basement membrane penetration *in vitro*. Here, we applied neutrophils from WT and NE^{-/-} mice, obtained from the peritoneum after thioglycollate injection, to a Matrigel invasion

Figure 3. NE^{-/-} mice developed increased extravascular lung water after mechanical ventilation as compared with wild-type controls. Wet:dry ratio of the left lung of WT (solid bars) and NE^{-/-} (shaded bars) mice ($n = 5$) after a 3-hour ventilation at varying tidal volumes was determined by incubating lungs in a 72°C oven for 24 hours. Data are presented as means $\pm$ SD. Wet:dry ratios, and hence lung injury, were significantly increased ($*P < 0.05$) in NE^{-/-} as compared with WT mice at both 20 ml/kg and 30 ml/kg tidal volumes.

assay. We found no difference in the numbers of WT and NE^{-/-} neutrophils that penetrated the Matrigel basement membrane matrix in response to neutrophil chemokines (Figure 5B). This is consistent with most previous studies that failed to inhibit neutrophil movement with NE inhibitors.

Penetration of WT and NE^{-/-} Neutrophils across hPVEC- and hPMVEC-Coated Membranes

The ability of neutrophils to cross pulmonary vein and microvascular endothelium *in vitro* was determined using a Transwell assay. In response to fMLP, WT neutrophils penetrated the endothelial barriers much more efficiently than NE^{-/-} neutrophils (hPVEC: 143.26 ± 10 and 16.8 ± 15.6 neutrophils/hpf for WT and NE^{-/-}, respectively [$P < 0.05$], and hPMVEC: 55.7 ± 16.2 and 27.4 ± 5.3 neutrophils/hpf for WT and NE^{-/-}, respectively [$P < 0.05$]). In contrast, penetration of the endothelial microvasculature was similar in WT and NE^{-/-} neutrophils in response to ZAS (Figure 5C).

Ability of WT and NE^{-/-} Neutrophils to Cause Endothelial Cell Injury upon Prolonged Contact and Transmigration

The ability of neutrophils to cross pulmonary endothelium and cause cellular injury *in vitro* was determined using a radioactive tracer ($\text{Na}^{51}\text{CrO}_4$). Neutrophils co-incubated with endothelial cells loaded with radioactive ^{51}Cr in a 96-well tissue chamber plate caused more injury in WT than in NE^{-/-} mice, as indicated by release of ^{51}Cr into the conditioned media, suggesting that NE-containing neutrophils, when in contact with endothelial cells, cause more injury than in its absence (Figure 6A). However, when WT and NE^{-/-} neutrophils are allowed to migrate through the endothelial barriers in response to fMLP, neutrophils from NE^{-/-} mice penetrated the endothelial barrier less effectively than neutrophils derived from WT mice and caused increased cellular injury as compared with WT (Figure 6B). The findings of increased endothelial cell injury upon prolonged exposure to NE^{-/-} neutrophils may explain the worsening lung injury (increased lung edema and lower static compliance) associated with the NE^{-/-} mice.

Capacity of NE^{-/-} and WT Neutrophils to Cleave Endothelial Surface ICAM-1

We hypothesized that the enhanced migration of WT as compared with NE^{-/-} neutrophils through endothelial cell barriers

Figure 4. Polymorphonuclear leukocyte (PMN) migration from the pulmonary vasculature into the alveolar space after mechanical ventilation is impaired in NE^{-/-} mice. (A.) To assess neutrophil migration from the vasculature into the alveolar space, neutrophils were stained with Gr-1 and neutrophils within the vasculature and within the alveoli were quantified by counting 50 random high-power fields per slide. Solid bars, WT mice; shaded bars, NE^{-/-} mice. (B) Representative Gr-1-stained sections for neutrophils are shown ($\times 40$). Note that WT neutrophils were more efficient than NE^{-/-} neutrophils in egressing from the vasculature into the alveolar spaces at 20 ml/kg (A) and 30 ml/kg tidal volumes ($*P < 0.05$). Arrowheads represent intravascular, and arrows represent alveolar neutrophils.

was due to NE-mediated cleavage of ICAM-1, releasing the neutrophil from the endothelial cell. To test this, we incubated activated WT and NE^{-/-} neutrophils with activated endothelial cells for 2 hours and assessed endothelial surface ICAM-1 expression using both Western blot analysis and immunofluorescence with an antibody raised against the extracellular domain of ICAM-1 (Figure 7). Despite greater numbers of adherent NE^{-/-} neutrophils in contact with endothelial cells (Figure 7A), there was much less cleavage of ICAM-1 observed both by Western blot (Figure 7B) and immunofluorescence (Figure 7C) by NE^{-/-} neutrophils as compared with WT neutrophils. Impaired NE-mediated cleavage of ICAM-1 would explain the prolonged neutrophil-endothelial cell contact leading to endothelial cell injury and increased permeability observed in NE^{-/-} mice.

DISCUSSION

This study demonstrates that after the onset of mechanical ventilation, mice developed decreased lung compliance and accumulated lung water in proportion to tidal volumes and time on the ventilator. In the absence of NE, mice developed worse parameters of lung injury than controls, particularly at higher tidal volumes and increased ventilator times. NE^{-/-} mice had a greater proportion of neutrophils “stuck” in the endothelial compartment as opposed to WT, where more

Figure 5. Capacity of WT (solid bars) and NE^{-/-} (shaded bars) neutrophils to migrate in response to chemotactic agents, invade extracellular matrix, and cross endothelial barriers. (A) Ability of WT and NE^{-/-} neutrophils to respond to chemotactic stimuli was measured using Boyden chamber assays. (B) WT and NE^{-/-} neutrophils were also tested for their capacity to invade the basement membrane substrate Matrigel in response to chemokines. (C) The capacity of WT and NE^{-/-} neutrophils to migrate through endothelial cell barriers in response to chemokines was also measured. Note that WT and NE^{-/-} neutrophils had similar capacity to respond to chemokines and to invade extracellular matrices. However, NE^{-/-} neutrophils were less capable than WT neutrophils of penetrating endothelial cells that were activated by the chemokine formyl-Met-Leu-Phe (fMLP) (but not zymosan). In addition to pulmonary vein migration (C), neutrophil migration through microvascular endothelial cells using fMLP at 1×10^{-5} were 55.7 ± 16.2 and 27.4 ± 5.3 PMN/hpf for WT and NE^{-/-}, respectively (* $P < 0.05$).

neutrophils did egress into alveolar spaces. Previous studies in cell culture revealed that presence or absence of NE did not influence the ability of neutrophils to respond to chemotactic signals or traverse extracellular matrix barriers (14). However,

in this study, in the presence of intact endothelium, WT neutrophils migrated through the endothelial barrier in response to chemotactic signals much more efficiently than NE^{-/-} neutrophils. This was likely related to NE-mediated cleavage of the

Figure 6. Neutrophil injury to endothelial cells upon prolonged contact. Endothelial cells intracellularly loaded with radioactive ^{51}Cr were either co-incubated with WT and NE^{-/-} neutrophils for 18 hours (A), or were allowed to migrate through an endothelial cell barrier grown on a microporous support membrane toward a chemotactic stimulus (B). Note that prolonged contact correlated with cell injury as measured by release of ^{51}Cr into the conditioned medium. WT neutrophils cause more injury than NE^{-/-} neutrophils, suggesting a destructive role for NE (A). However, if allowed to migrate through the endothelial barrier, NE^{-/-} neutrophils, whose penetration is impaired cause more injury than WT with significantly higher release of ^{51}Cr (* $P < 0.05$) (B).

firm attachment of the neutrophil to ICAM-1 allowing neutrophils to egress through the endothelium. Prolonged contact of activated neutrophils with endothelial cells results in cell injury, as demonstrated by chromium release assays. In fact, there was greater cell injury when neutrophils contained NE than in its absence. However, when allowed to traverse the endothelial barrier in

response to chemotactic stimuli, there were fewer WT as compared with NE^{-/-} neutrophils in contact with endothelial cells and less endothelial cell injury. Proteases and reactive oxygen species in addition to NE could injure the endothelium, which may explain our findings (19–21). These results suggest a role for NE in facilitating egression of neutrophils from the vasculature

Figure 7. Increased cleavage of endothelial intercellular adhesion molecule (ICAM)-1 by WT as compared with NE^{-/-} neutrophils. Activated endothelial cells were co-incubated with activated WT and NE^{-/-} neutrophils. (A) NE^{-/-} neutrophils adhered more avidly to the endothelium at the end of 2 hours of incubation than did WT neutrophils. Yet, despite less adherence, WT neutrophils cleaved endothelial surface ICAM-1 more efficiently than NE^{-/-} neutrophils as shown in the Western blot (B) and immunofluorescence (C).

into alveolar spaces during mechanical ventilation in mice, and that continued contact of neutrophils with endothelial cells could lead to lung injury.

Neutrophil elastase is a 28-kD serine proteinase, characterized by conserved His, Asp, Ser residues that form a charge relay system resulting in a powerful nucleophile able to cleave peptide bonds of the substrate (22). Neutrophil serine proteinases are only expressed during the myelomonocytic stage of leukocyte development, and are stored for later use as active enzymes within the neutrophil's primary granules. Upon activation, neutrophils release approximately 2% of their NE content into the extracellular space, where they can reach high local concentrations overwhelming inhibitors transiently (23). In addition, about 12% of NE content is mobilized to the cell membrane, where it is catalytically active and resistant to inhibitors. Cell surface NE is strategically positioned for cell-cell and cell-matrix interactions and localized extracellular proteolysis (23).

NE has a broad range of susceptible extracellular matrix substrates including elastin, interstitial collagens (I-III), and basement membrane proteins (24). Other relevant substrates include coagulation factors, fibronectin, plasminogen, immunoglobulins, and C5a (25, 26). Hence, NE has been thought to play a role in a variety of destructive inflammatory diseases including ARDS (27). NE has also been postulated to "clear paths" through extracellular and promote neutrophil trafficking, but as discussed above few studies support this concept.

We and others previously generated NE^{-/-} mice, which helped establish a role for NE in host defense. Specifically, NE is required for intracellular neutrophil killing Gram-negative bacteria via degradation of outer membrane proteins (Omps) (15, 28, 29). Neutrophil function was otherwise normal; however, the role of NE in neutrophil migration assays within the context of VILI was not previously reported. Application of NE^{-/-} mice to a model of cigarette smoke-induced emphysema demonstrated that NE contributed to monocyte, but not neutrophil, recruitment into the lung and was in part responsible for airspace enlargement (30). In response to bleomycin, there were no differences in neutrophil inflammation, but NE^{-/-} mice were also protected from fibrosis secondary to NE-mediated activation of TGF- β (31).

In this study, to determine the role of NE in VILI, we applied NE^{-/-} mice to mechanical ventilation. Beginning with classic studies of mechanical ventilation in dog models, and extending to rodents, investigators describe increased numbers of intra-alveolar and intravascular leukocytes and macrophages after VILI using high tidal volumes (1, 32). High tidal volumes in animal models of VILI have been criticized for not reflecting the actual practice at bedside, but these Tv (20–30 ml/kg) are comparable to the excessive overdistention expected in some parts of the lungs in ARDS due to its heterogeneous nature (5). We used a volume-based lung injury model along with 0 cm H₂O of PEEP to induce VILI. Surprisingly, in the absence of NE, mice developed worse injury than when this potentially destructive enzyme was present.

NE's beneficial role in VILI could relate to its requirement for neutrophils to egress from the pulmonary vasculature. In the absence of NE, prolonged endothelial cell-neutrophil contact led to endothelial cell injury and increased permeability, similar to a previous report on neutrophil-epithelial cell interaction (33). The role of NE in neutrophil trafficking remains of great interest and controversial. As discussed, most cell culture studies failed inhibit NE migration using NE inhibitors (14, 34). However, there are published studies showing inhibition of NE resulting in diminished neutrophil chemotaxis (35) and matrix (Matrigel) invasion (36). This study shows that upon the addition of an endothelial cell barrier, NE is required for transvascular

egression. The effect of NE on neutrophil trafficking *in vivo* varies depending upon the model. In some studies of acute lung injury, NE inhibitors (endogenous, small molecular weight compounds, or antibodies) or lack of NE ameliorate neutrophil accumulation *in vivo* (35, 37, 38). Yet, application of NE^{-/-} mice to other animal models such as bleomycin and emphysema (above), as well as lipopolysaccharide (16), did not demonstrate a role for NE in neutrophil accumulation in the lung.

We performed further experiments to determine the mechanism by which NE promotes transvascular migration in the context of an activated pulmonary endothelium. First, we observed that NE-deficient neutrophils as compared with WT neutrophils had impaired migration through endothelial monolayers in response to fMLP, but not zymosan. Since fMLP, but not zymosan, induces endothelial ICAM-1 and displays NE-mediated neutrophil migration, this suggested a role for NE in β_2 -integrin-mediated migration. Previously, NE has been shown to release neutrophils from endothelial cells noncatalytically by interfering with CD11b/CD18-ICAM-1 interactions (39). Yet, NE was also shown to catalytically cleave ICAM-1 on monocytic cell lines (39, 40). Our data suggest a role for NE in catalytically disrupting ICAM-1-CD11b/CD18 bonds releasing neutrophils from endothelial cells, allowing them to follow the chemotactic gradient.

Despite the fact that NE itself is an injurious proteinase, the NE-deficient neutrophil contains a variety of toxic oxidants and other proteinases that can harm endothelial cells upon prolonged contact. Hence, while WT neutrophils are more harmful to endothelial cells than NE^{-/-} neutrophils, it is in the absence of NE that they have prolonged contact and hence cause cell injury.

This study suggests that retention of neutrophils within pulmonary vasculature has an acute injurious effect on the lung in mice. Whether the detrimental effects of NE-containing neutrophils within the alveolar space ultimately have more severe consequences on lung injury over time is not known. However, it is interesting to note in the context of ARDS that a clinical trial using small-molecular-weight NE inhibitor resulted in a negative trend in long-term mortality, with no superiority in ventilator-free days or 28-day all-cause mortality over placebo (41). Whether this is a function of NE's antimicrobial activity or its role in neutrophil egress, the mechanisms invoked here or others, is not known (15). Given NE's physiological functions to release neutrophils from the vasculature and kill bacteria within the neutrophil, perhaps an ideal inhibitor would be one that did not penetrate the neutrophil and introduced to (and limited to) the alveolar space. These findings also reinforce the importance of limiting mechanical ventilation volumes with consequent reduction in inflammation contributing to volutrauma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Tremblay L, Valenza F, Ribeiro SP, Li J, Slutsky AS. Injurious ventilatory strategies increase cytokines and c-fos m-rna expression in an isolated rat lung model. *J Clin Invest* 1997;99:944–952.
2. Chapman KE, Sinclair SE, Zhuang D, Hassid A, Desai LP, Waters CM. Cyclic mechanical strain increases reactive oxygen species production in pulmonary epithelial cells. *Am J Physiol* 2005;289:L834–L841.
3. Belperio JA, Keane MP, Burdick MD, Londhe V, Xue YY, Li K, Phillips RJ, Strieter RM. Critical role for cxcr2 and cxcr2 ligands during the pathogenesis of ventilator-induced lung injury. *J Clin Invest* 2002;110:1703–1716.
4. Imai Y, Parodo J, Kajikawa O, de Perrot M, Fischer S, Edwards V, Cutz E, Liu M, Keshavjee S, Martin TR, *et al.* Injurious mechanical ventilation and end-organ epithelial cell apoptosis and organ dysfunction in an experimental model of acute respiratory distress syndrome. *JAMA* 2003;289:2104–2112.

5. Gattinoni L, Presenti A, Torresin A, Baglioni S, Rivolta M, Rossi F, Scarani F, Marcolin R, Cappelletti G. Adult respiratory distress syndrome profiles by computed tomography. *J Thorac Imaging* 1986;1: 25–30.
6. The acute respiratory distress syndrome network. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. *N Engl J Med* 2000;342:1301–1308.
7. Markos J, Doerschuk CM, English D, Wiggs BR, Hogg JC. Effect of positive end-expiratory pressure on leukocyte transit in rabbit lungs. *J Appl Physiol* 1993;74:2627–2633.
8. Inano H, English D, Doerschuk CM. Effect of zymosan-activated plasma on the deformability of rabbit polymorphonuclear leukocytes. *J Appl Physiol* 1992;73:1370–1376.
9. Wang Q, Pfeiffer GR II, Stevens T, Doerschuk CM. Lung microvascular and arterial endothelial cells differ in their responses to intercellular adhesion molecule-1 ligation. *Am J Respir Crit Care Med* 2002;166: 872–877.
10. Doerschuk CM, Winn RK, Coxson HO, Harlan JM. Cd18-dependent and -independent mechanisms of neutrophil emigration in the pulmonary and systemic microcirculation of rabbits. *J Immunol* 1990;144: 2327–2333.
11. Kumasaka T, Doyle NA, Quinlan WM, Graham L, Doerschuk CM. Role of cd 11/cd 18 in neutrophil emigration during acute and recurrent pseudomonas aeruginosa-induced pneumonia in rabbits. *Am J Pathol* 1996;148:1297–1305.
12. Willeke T, Scharffetter-Kochanek K, Gaetgens P, Walzog B. A role for beta2 integrin (cd11/cd18)-mediated tyrosine signaling in extravasation of human polymorphonuclear neutrophils. *Biorheology* 2001;38: 89–100.
13. Lee CT, Fein AM, Lippmann M, Holtzman H, Kimbel P, Weinbaum G. Elastolytic activity in pulmonary lavage fluid from patients with adult respiratory-distress syndrome. *N Engl J Med* 1981;304:192–196.
14. Huber AR, Weiss SJ. Disruption of the subendothelial basement membrane during neutrophil diapedesis in an in vitro construct of a blood vessel wall. *J Clin Invest* 1989;83:1122–1136.
15. Belaouaj A, McCarthy R, Baumann M, Gao Z, Ley TJ, Abraham SN, Shapiro SD. Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. *Nat Med* 1998;4:615–618.
16. Hirche TO, Atkinson JJ, Bahr S, Belaouaj A. Deficiency in neutrophil elastase does not impair neutrophil recruitment to inflamed sites. *Am J Respir Cell Mol Biol* 2004;30:576–584.
17. Mizgerd JP, Kubo H, Kutkoski GJ, Bhagwan SD, Scharffetter-Kochanek K, Beaudet AL, Doerschuk CM. Neutrophil emigration in the skin, lungs, and peritoneum: different requirements for cd11/cd18 revealed by cd18-deficient mice. *J Exp Med* 1997;186:1357–1364.
18. Horwitz M, Benson KF, Person RE, Aprikan AG, Dale DC. Mutations in ela2, encoding neutrophil elastase, define a 21-day biological clock in cyclic haematopoiesis. *Nat Genet* 1999;23:433–436.
19. Guice KS, Oldham KT, Caty MG, Johnson KJ, Ward PA. Neutrophil-dependent, oxygen-radical mediated lung injury associated with acute pancreatitis. *Ann Surg* 1989;210:740–747.
20. Till GO, Johnson KJ, Kunkel R, Ward PA. Intravascular activation of complement and acute lung injury: dependency on neutrophils and toxic oxygen metabolites. *J Clin Invest* 1982;69:1126–1135.
21. Tumen J, Meyrick B, Berry L Jr, Brigham KL. Antiproteinases protect cultured lung endothelial cells from endotoxin injury. *J Appl Physiol* 1988;65:835–843.
22. Bode W, Meyer E Jr, Powers JC. Human leukocyte and porcine pancreatic elastase: X-ray crystal structures, mechanism, substrate specificity, and mechanism-based inhibitors. *Biochemistry* 1989;28: 1951–1963.
23. Owen CA, Campbell MA, Sannes PL, Boukedes SS, Campbell EJ. Cell surface-bound elastase and cathepsin g on human neutrophils: a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. *J Cell Biol* 1995;131:775–789.
24. Mainardi CL, Dixit SN, Kang AH. Degradation of type iv (basement membrane) collagen by a proteinase isolated from human polymorphonuclear leukocyte granules. *J Biol Chem* 1980;255:5435–5441.
25. DiMartino SJ, Shah AB, Trujillo G, Kew RR. Elastase controls the binding of the vitamin d-binding protein (gc-globulin) to neutrophils: a potential role in the regulation of c5a co-chemotactic activity. *J Immunol* 2001;166:2688–2694.
26. McDonald JA, Kelley DG. Degradation of fibronectin by human leukocyte elastase: release of biologically active fragments. *J Biol Chem* 1980; 255:8848–8858.
27. Shapiro SD. Neutrophil elastase: path clearer, pathogen killer, or just pathologic? *Am J Respir Cell Mol Biol* 2002;26:266–268.
28. Tkaleciv J, Novelli M, Phylactides M, Iredale JP, Segal AW, Roes J. Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin g. *Immunity* 2000;12: 201–210.
29. Belaouaj A, Kim KS, Shapiro SD. Degradation of outer membrane protein a in Escherichia coli killing by neutrophil elastase. *Science* 2000;289:1185–1188.
30. Shapiro SD, Goldstein NM, Houghton AM, Kobayashi DK, Kelley D, Belaouaj A. Neutrophil elastase contributes to cigarette smoke-induced emphysema in mice. *Am J Pathol* 2003;163:2329–2335.
31. Chua F, Dunsmore SE, Clingen PH, Mutsaers SE, Shapiro SD, Segal AW, Roes J, Laurent GJ. Mice lacking neutrophil elastase are resistant to bleomycin-induced pulmonary fibrosis. *Am J Pathol* 2007;170: 65–74.
32. Woo SW, Hedley-Whyte J. Macrophage accumulation and pulmonary edema due to thoracotomy and lung over inflation. *J Appl Physiol* 1972;33:14–21.
33. Parsons PE, Sugahara K, Cott GR, Mason RJ, Henson PM. The effect of neutrophil migration and prolonged neutrophil contact on epithelial permeability. *Am J Pathol* 1987;129:302–312.
34. Woodman RC, Reinhardt PH, Kanwar S, Johnston FL, Kubes P. Effects of human neutrophil elastase (hne) on neutrophil function in vitro and in inflamed microvessels. *Blood* 1993;82:2188–2195.
35. Delacourt C, Herigault S, Delclaux C, Poncin A, Levame M, Harf A, Saudubray F, Lafuma C. Protection against acute lung injury by intravenous or intratracheal pretreatment with epi-hne-4, a new potent neutrophil elastase inhibitor. *Am J Respir Cell Mol Biol* 2002;26:290–297.
36. Delclaux C, Delacourt C, D'Ortho MP, Boyer V, Lafuma C, Harf A. Role of gelatinase b and elastase in human polymorphonuclear neutrophil migration across basement membrane. *Am J Respir Cell Mol Biol* 1996;14:288–295.
37. Honore S, Attalah HL, Azoulay E, Soussy CJ, Saudubray F, Harf A, Brochard L, Delclaux C. Beneficial effect of an inhibitor of leukocyte elastase (epi-hne-4) in presence of repeated lung injuries. *Shock* 2004; 22:131–136.
38. Wang S, Dangerfield JP, Young RE, Nourshargh S. Pecam-1, alpha6 integrins and neutrophil elastase cooperate in mediating neutrophil transmigration. *J Cell Sci* 2005;118:2067–2076.
39. Cai TQ, Wright SD. Human leukocyte elastase is an endogenous ligand for the integrin cr3 (cd11b/cd18, mac-1, alpha m beta 2) and modulates polymorphonuclear leukocyte adhesion. *J Exp Med* 1996; 184:1213–1223.
40. Champagne B, Tremblay P, Cantin A, St Pierre Y. Proteolytic cleavage of icam-1 by human neutrophil elastase. *J Immunol* 1998;161:6398–6405.
41. Zeiher BG, Artigas A, Vincent JL, Dmitrienko A, Jackson K, Thompson BT, Bernard G. Neutrophil elastase inhibition in acute lung injury: results of the strive study. *Crit Care Med* 2004;32:1695–1702.