Procoagulant alveolar microparticles in the lungs of patients with acute respiratory distress syndrome

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Bastarache JA, Fremont RD, Kropski JA, Bossert FR, Ware LB. Procoagulant alveolar microparticles in the lungs of patients with acute respiratory distress syndrome. Am J Physiol Lung Cell Mol Physiol 297: L1035–L1041, 2009. First published August 21, 2009; doi:10.1152/ajplung.00214.2009.—Coagulation and fibrinolysis abnormalities are observed in acute lung injury (ALI) in both human disease and animal models and may contribute to ongoing inflammation in the lung. Tissue factor (TF), the main initiator of the coagulation cascade, is upregulated in the lungs of patients with ALI/acute respiratory distress syndrome (ARDS) and likely contributes to fibrin deposition in the air space. The mechanisms that govern TF upregulation and activation in the lung are not well understood. In the vascular space, TF-bearing microparticles (MPs) are central to clot formation and propagation. We hypothesized that TF-bearing MPs in the lungs of patients with ARDS contribute to the procoagulant phenotype in the air space during acute injury and that the alveolar epithelium is one potential source of TF MPs. We studied pulmonary edema fluid collected from patients with ARDS compared with a control group of patients with hydrostatic pulmonary edema. Patients with ARDS have higher concentrations of MPs in the lung compared with patients with hydrostatic edema (25.5 IQR 21.3–46.9 vs. 7.8 IQR 2.3–27.5 μmol/l, P = 0.009 by Mann-Whitney U-test). These MPs are enriched for TF, have procoagulant activity, and likely originate from the alveolar epithelium [as measured by elevated levels of RAGE (receptor for advanced glycation end products) in ARDS MPs compared with hydrostatic MPs]. Furthermore, alveolar epithelial cells in culture release procoagulant TF MPs in response to a proinflammatory stimulus. These findings suggest that alveolar epithelial-derived MPs are one potential source of TF procoagulant activity in the air space in ARDS and that epithelial MP formation and release may represent a unique therapeutic target in ARDS.

coagulation; fibrin deposition; tissue factor; receptor for advanced glycation end products; hydrostatic pulmonary edema

ACTIVATION OF COAGULATION in the alveolar compartment is central to the pathogenesis of acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) (22, 25), but the specific mechanisms that regulate coagulation proteins in the lung are unknown. We recently reported that the alveolar epithelium is a key modulator of intra-alveolar coagulation, responding to inflammatory stimuli with upregulation of cell surface tissue factor (TF) procoagulant activity (5) and decreased capacity to generate activated protein C (24). In vitro, alveolar epithelial cells upregulate cell surface TF activity in response to a proinflammatory stimulus. Pulmonary edema fluid from patients with ALI/ARDS is highly enriched with TF protein and TF-dependent procoagulant activity compared with plasma from the same patients (5). These prior findings suggest that the lung epithelium can modulate intra-alveolar coagulation and fibrin deposition both through cell surface TF expression and through release of active TF.

In the vascular space, the regulation of TF activity is complex, involving not only upregulation of TF on the surface of endothelial cells and macrophages but also release of TF-containing microparticles (MPs) that participate actively in clot formation. MPs are membrane-bound particles that are less than 1 μm in size and are produced by several cell types (macrophages, platelets, endothelial cells) following an inflammatory or apoptotic stimulus. Procoagulant MPs are central to the revised theory of coagulation (15). Upon activation, platelets (26), monocytes, and endothelial cells (2) release TF-bearing procoagulant MPs that amplify thrombin and fibrin formation leading to clot formation and propagation. MPs also participate in antigen presentation through the major histocompatibility complex (16, 17), in activation of inflammatory cells (14), and can transfer membrane-bound proteins from one cell to another (19). The formation and release of MPs, once thought to be merely a byproduct of cell activation or injury, is now understood to be a highly regulated process.

Early studies showed that bronchoalveolar lavage fluid from patients with ARDS contained sedimentable (at 100,000 g) TF procoagulant activity, suggesting that TF in the air space may be in MPs, but further characterization was not done in this study (10). Given the critical importance of MP-associated TF in systemic coagulation, we hypothesized that TF-bearing MPs are important in intra-alveolar coagulation and fibrin deposition in the acutely injured lung. Furthermore, we hypothesized that the alveolar epithelium is one potential source of TF-bearing MPs. To address these questions, we characterized the MP-associated procoagulant activity in undiluted pulmonary edema fluid from a group of critically ill patients with ALI/ARDS compared with a control group of critically ill patients with hydrostatic (cardiogenic) pulmonary edema. In addition, we measured MP release from cytokine-stimulated lung epithelial cells (A549 cells) to determine whether lung epithelial cells are a potential source for procoagulant intra-alveolar MPs.

MATERIALS AND METHODS

Patients. Intubated and mechanically ventilated patients at Vanderbilt University Medical Center were enrolled in the study if they met the following criteria: acute onset of respiratory failure requiring
intubation and mechanical ventilation accompanied by hypoxemia and bilateral infiltrates on chest imaging. Patients were classified as having ALI/ARDS based on the consensus definition of 1) bilateral infiltrates on chest radiograph, 2) hypoxemia, and 3) absence of left heart failure (1). Hydrostatic (cardiogenic) pulmonary edema was defined as bilateral infiltrates and evidence of left heart failure (23). Pulmonary edema fluid was collected within 6 h of intubation as previously described (12). Briefly, a standard suction catheter was inserted through the endotracheal tube and advanced slowly until meeting resistance. Gentle suction was applied, and a few milliliters of pulmonary edema fluid was aspirated. Samples were placed immediately on ice and centrifuged at 500 g for 10 min to remove cellular components, and the supernatant was stored at −70°C until use. In addition, demographic and clinical data were collected on each patient. The variables collected included age, gender, race, cause of pulmonary edema, ventilator settings, SpO2/FiO2 ratio (18), and hospital mortality. Radiographic and laboratory data were reviewed, and Lung Injury Score (LIS) (13) and Simplified Acute Physiology Score (SAPS II) (11) were calculated for each patient. The study was approved with a waiver of consent by the Institutional Review Board at Vanderbilt University Medical Center.

Electron microscopy. Edema fluid was prepared for electron microscopy (EM) by sucrose gradient separation. A sucrose gradient (30%, 40%, 50%, 60% sucrose, respectively) was prepared, and 100 μl of edema fluid was layered on top and centrifuged at 100,000 g (4°C) for 18 h. The fractions were collected in 250-μl aliquots. To determine the peak concentration of MPs, TF protein was measured by ELISA (American Diagnostica, Stamford, CT) and was highest at the interface between 30% and 40% sucrose. A drop of this fraction was placed on a metal EM grid and allowed to air dry for 30 s. The sample was negatively stained with a 1% solution of phosphotungstic acid for 30 s. A Philips CM12 TEM (transmission electron microscope) and an AMT 542 digital camera with 1,064 × 1,064 digital resolution were used to acquire images.

Differential centrifugation of edema fluid. To separate edema fluid into MPs and soluble factors, edema fluid samples were thawed and subject to three centrifugation steps at 4°C. For each sample, 100 μl of edema fluid was diluted in 900 μl of sterile filtered PBS. First, samples were centrifuged at 1,500 g for 20 min to remove any large debris that may have formed during storage. The pellet was discarded. Next, the samples were spun at 20,000 g for 30 min to remove platelets and smaller cellular debris (20). The supernate was then centrifuged at 100,000 g to pellet MPs that were resuspended in 1,000 μl of PBS (3). The supernatant of the final centrifugation was reserved and contained all of the soluble factors in edema fluid. All samples were stored at −70°C until measurements were made.

MP capture assay. MP concentration was measured in MP fractions from edema fluid and cell culture with a microplate-based MP capture and activity assay (Hyphen Biomed, Neuville sur Oise, France) (4). Microtiter plates were coated with streptavidin and biotinylated annexin V. When a sample is added, the membrane particles bind to the annexin V and are captured on the microtiter plate. The exposed phospholipids (MPs) are incubated with activated coagulation factors V and X and prothrombin. Thrombin generation is measured using a chromogenic substrate. In the presence of active factor V and X, the limiting step in thrombin generation is the microtiter plate-bound phospholipid concentration. Absolute MP concentrations are derived from a standard curve using a MP calibrator from the manufacturer. Measurements were done in duplicate.

Cell culture. A549 cells (American Type Culture Collection) were grown in Minimal Essential Media (Cellgro, Manassas, VA) to confluence in 24-well plates, washed twice with 0.5 ml of MEM without FBS, and exposed to 20 ng/ml cytokinin (TNF-α, IL-1β, IFN-γ) or control medium (MEM without FBS) for 0–24 h. After the designated amount of time, conditioned media were collected and centrifuged at 500 g for 10 min to remove large cellular debris. The supernatants were stored at −70°C until differential centrifugation as described above.

Clot time measurement. Clot time was measured using a mechanical clot detection system (STart4 Coagulometer; Diagnostica Stago, Asnieres, France). Briefly, 50 μl of MPs or soluble fraction from edema fluid or cell culture-conditioned media was warmed for 15 min at 37°C. Samples were then incubated with 50 μl of pooled normal human plasma (Fisher Diagnostics, Middletown, VA) for 2 min. Clot time was determined as recalcification time following the addition of 50 μl of 50 mM calcium chloride. Measurements were done in duplicate.

Protein, TF, and RAGE measurements. Protein was measured in all samples using the BCA protein assay kit (Pierce, Rockford, IL). TF (American Diagnostica) and RAGE (R&D Systems, Minneapolis, MN) were measured according to the manufacturers’ instructions. All measurements were performed in duplicate.

Statistical methods. Statistical analysis was done using SPSS software version 13.0 for Macintosh. Differences between two groups were assessed by Mann-Whitney U-test, whereas differences between multiple groups were assessed by ANOVA with post hoc Tukey test or Bonferroni correction. Proportions were compared using the Fisher exact test. For all analyses, \( P < 0.05 \) was considered statistically significant.

RESULTS

Patient characteristics. Clinical characteristics of all patients are shown in Table 1. Patients with ARDS tended to be younger than those with hydrostatic edema. ARDS patients had a higher edema fluid-to-plasma protein ratio consistent with increased permeability pulmonary edema. In addition, they had a higher LIS. Both groups were ventilated with a low tidal volume strategy, and both patient groups were critically ill with similarly high SAPS II scores. Mortality was similar in both groups, 38% in the hydrostatic group and 36% in the ARDS group.

EM of ARDS MPs. MPs in edema fluid from a patient with ARDS were analyzed by negative staining EM (Fig. 1). Multiple spherical particles ranging in diameter from 32 to 59 nm were seen in the sucrose gradient fraction that contained the highest concentration of TF. By contrast, a fraction of the sucrose gradient that had undetectable levels of TF did not contain MPs by EM.

MP concentrations are higher in patients with ARDS compared with patients with hydrostatic edema. To quantify membrane MPs in the 100,000 g fraction of edema fluid after

<table>
<thead>
<tr>
<th>Table 1. Clinical characteristics of patients</th>
<th>Hydrostatic ( (n=13) )</th>
<th>ARDS ( (n=11) )</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>50 (33–58)</td>
<td>34 (23–47)</td>
<td>0.17</td>
</tr>
<tr>
<td>Lung Injury Score</td>
<td>2.9 (2.3–3.7)</td>
<td>3.7 (3.0–3.8)</td>
<td>0.04</td>
</tr>
<tr>
<td>S/F ratio</td>
<td>100 (87–180)</td>
<td>115 (96–167)</td>
<td>0.28</td>
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<tr>
<td>EF/PL protein ratio</td>
<td>0.47 (0.33–0.55)</td>
<td>0.85 (0.77–1.06)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TV/kg</td>
<td>6.8 (5.4–7.8)</td>
<td>6.3 (4.9–6.6)</td>
<td>0.17</td>
</tr>
<tr>
<td>Simplified Acute Physiology Score II</td>
<td>41 (32–49)</td>
<td>40 (30–49)</td>
<td>0.78</td>
</tr>
<tr>
<td>Days of unassisted ventilation</td>
<td>21 (0.5–24)</td>
<td>14 (0–24)</td>
<td>0.28</td>
</tr>
<tr>
<td>Death n (%)</td>
<td>5 (38)</td>
<td>4 (36)</td>
<td>0.63</td>
</tr>
</tbody>
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Data are expressed as median (IQR) except as otherwise noted. Lung Injury Score (13); S/F ratio, SpO2/FiO2 ratio (18); EF, edema fluid; PL, plasma; TV/kg, tidal volume corrected for actual body weight; Simplified Acute Physiology Score II (11).
differential centrifugation, we measured MP concentration by MP capture assay. Patients with ARDS had significantly higher MP concentrations in edema fluid compared with those with hydrostatic edema (Fig. 2).

**Edema fluid fraction characteristics.** Protein concentrations were measured in all three edema fluid fractions (20,000 g pellet, soluble factors, and MPs). The majority of protein in both ARDS and hydrostatic pulmonary edema fluid was found in the soluble fraction compared with the two membrane-containing fractions (Fig. 3) and in both patient groups was >100-fold higher in the soluble fraction compared with the other two fractions. In all fractions, total protein was higher in patients with ARDS than in patients with hydrostatic edema.

Fig. 1. Negative staining electron microscopy of microparticles (MPs) from edema fluid of a patient with acute respiratory distress syndrome (ARDS). A: low magnification (direct magnification, ×53,000) of edema fluid before sucrose gradient separation. Arrows indicate MPs of varying sizes. B and C: a low-magnification (×53,000) view of the same edema fluid sample following sucrose gradient separation. B was taken from a gradient fraction with undetectable tissue factor (TF) protein, whereas C was from a fraction with high TF protein content. Arrows indicate MPs of uniform size. D: a higher magnification (direct magnification, ×230,000) of C. MPs shown in D are 32–59 nm in diameter.

Fig. 2. Boxplot of the MP concentration in the MP fraction as measured by MP capture assay in the edema fluid of patients with hydrostatic pulmonary edema compared with those with ARDS. Horizontal bars, median; box, 25–75th percentile; error bars, 10–90th percentile. $P = 0.009$ vs. hydrostatic by Mann-Whitney U-test.

Fig. 3. Protein concentration of edema fluid fractions in patients with hydrostatic edema and ARDS. In both groups, the protein concentration was 100-fold higher in the soluble fraction compared with either the 20,000 g pellet or the MPs. In all fractions, protein concentration was higher in ARDS vs. hydrostatic, but the difference did not reach statistical significance. $*P < 0.001$ compared with 20,000 g pellet and MPs by ANOVA with post hoc Bonferroni correction.
Edema fluid procoagulant activity is higher in MP fraction and increased in patients with ARDS. We have previously reported increases in TF procoagulant activity in edema fluid from patients with ARDS (5). To define the distribution of procoagulant activity in edema fluid, we measured the clot time in the soluble fraction compared with the MP fraction in all patients. Increased procoagulant activity, as measured by a shortening of the clot time, was present in the MP fraction compared with the soluble fraction in patients with ARDS (Fig. 4). Additionally, MPs from ARDS patients had significantly increased procoagulant activity compared with MPs from patients with hydrostatic pulmonary edema.

Procoagulant MPs are enriched for TF. To determine if increased procoagulant activity of MPs correlated with levels of TF protein, we measured TF protein by ELISA in the MP fraction and the soluble fraction of edema fluid from patients with ARDS compared with patients with hydrostatic pulmonary edema (Fig. 5). Despite the low total protein levels in the MP fraction, the MPs were highly enriched for TF and had increased TF concentration compared with the soluble fraction. Furthermore, MPs from ARDS patients had significantly more TF compared with MPs from patients with hydrostatic edema. The relationship between TF and MP concentration in the MP fraction is shown in Fig. 6. There was a strong correlation between TF and MP concentration (Spearman correlation coefficient = 0.45, P = 0.028).

ARDS MPs have higher RAGE levels compared with hydrostatic MPs. To determine if a portion of intra-alveolar MPs was derived from lung epithelial cells, we measured an epithelial cell marker, the receptor for advanced glycation end products (RAGE), in MPs from patients with ARDS compared with those with hydrostatic edema (Fig. 7). MPs isolated from patients with ARDS had significantly higher RAGE levels compared with MPs from patients with hydrostatic edema.

Alveolar epithelial cells release procoagulant MPs in response to an inflammatory stimulus. To determine if cultured alveolar epithelial cells release procoagulant MPs when stimulated with a proinflammatory stimulus, we measured clot time, TF protein, and MP concentrations in the conditioned media of cytokine-stimulated alveolar epithelial cells (A549 cells). Conditioned media from A549 cells have increased procoagulant activity (Fig. 8A), TF levels (Fig. 8B), and MP concentration (Fig. 8C) over time in response to cytomix.

MP concentration is associated with mortality. There was a trend towards a higher MP concentration in the edema fluid from patients who died compared with patients who lived (Fig. 9), although this difference did not reach statistical significance. MP concentration, MP clot time, and MP TF concentration were not associated with clinical markers of mortality.

Fig. 4. Distribution of procoagulant activity in the alveolar compartment in patients with hydrostatic pulmonary edema compared with ARDS. Bars represent means ± SD. *P = <0.001 by Mann-Whitney U-test. **P = 0.007 by Mann-Whitney U-test.

Fig. 5. TF protein concentration in soluble fraction compared with MPs in edema fluid of patients with hydrostatic pulmonary edema compared with ARDS. *P = 0.015 by Mann-Whitney U-test. **P = 0.041 by Mann-Whitney U-test.

Fig. 6. Scatterplot of the MP concentration in edema fluid vs. the TF content of the MP fraction (both log transformed) in ARDS (triangles) vs. hydrostatic edema (circles). The Spearman correlation coefficient = 0.45, P = 0.028.

Fig. 7. Boxplot of RAGE concentration in MPs in patients with hydrostatic pulmonary edema compared with those with ARDS. ○, outlier; *P = 0.007 by Mann-Whitney U-test.
severity of illness (SAPS II), lung injury (LIS, SpO2/FIO2 ratio), or ventilator settings (tidal volume/kg).

**DISCUSSION**

In this study, we have shown that the alveolar compartment in patients with ARDS contains higher concentrations of procoagulant MPs compared with patients with hydrostatic edema. Furthermore, TF protein is highly concentrated in MPs despite the fact that the majority of protein in the air space is soluble and not MP associated. Although there are several possible cellular sources of intra-alveolar MPs, we have identified the alveolar epithelium as a potential source of procoagulant MPs.

Although the mechanisms of formation and function of MPs have been studied in the circulation and in several disease states, the role of MPs in ALI is unknown. In this study, we utilized a unique resource, pulmonary edema fluid from mechanically ventilated patients with ALI/ARDS, to characterize and study procoagulant MPs in the air space. Pulmonary edema is a complex biological fluid containing cell debris and fragments, soluble proteins, and MPs. To specifically study and characterize MPs, we developed a four-step centrifugation process to first remove intact cells (500 g for 10 min, then 1,500 g for 20 min), larger cell debris and platelets (20,000 g for 30 min), and finally to separate MPs and soluble factors (100,000 g for 60 min), resulting in two fractions for study: soluble factors and MPs.

We have previously reported that procoagulant activity in edema fluid is higher in ARDS compared with hydrostatic pulmonary edema (5) and that this increased procoagulant activity was TF dependent. However, we did not quantify MPs in these patients. In the current study, procoagulant activity (as measured by recalcification time) was higher in MPs compared with soluble factors in both hydrostatic pulmonary edema and ARDS. MPs from the air spaces of patients with ARDS were more procoagulant than those found in hydrostatic edema. However, even patients with hydrostatic edema had procoagulant MPs in the air space. All patients in this study, including those with hydrostatic edema, were critically ill, intubated, and mechanically ventilated. Although invasive ventilation is life-saving and necessary, recent studies have shown that even normal, uninjured lungs undergo activation of coagulation when

**Fig. 8.** Clot time (A), TF concentration (B), and MP concentration (C) of cell-free conditioned media from A549 cells treated for varying lengths of time with serum-free culture media (control) or 20 ng/ml cytomix. *P < 0.05 vs. 0-h cytomix, †P < 0.05 vs. 0-h control by ANOVA with post hoc Tukey test.

**Fig. 9.** Boxplot comparing MP concentrations in survivors (alive) vs. nonsurvivors (dead) in all patients. Patients who died had a trend towards a higher MP concentration compared with those who survived. P = 0.073 by Mann-Whitney U-test.
exposed to mechanical ventilation with high tidal volumes (8). Alterations in coagulation in response to mechanical ventilation may be more pronounced in critical illness and could explain the findings in patients with hydrostatic pulmonary edema. However, this hypothesis is speculative, and further studies of MP release in hydrostatic pulmonary edema and the effect of mechanical ventilation are needed.

To further characterize the procoagulant MPs, we measured TF antigen in both the soluble fraction and MPs in all patients. TF levels were higher in both fractions in ARDS patients compared with those with hydrostatic edema. Despite the 10-fold higher protein concentration in the soluble edema fluid fraction, TF protein levels were higher in the MPs than in the soluble fraction demonstrating that TF protein is highly concentrated in MPs. Other studies have shown that MP formation is a highly regulated process with cell surface TF being sequestered into lipid rafts that are then released as MPs (9). The high concentration of TF in MPs in ARDS edema fluid suggests that a similar process may be occurring in the lung during intra-alveolar MP release, but further studies are needed to more carefully define this process. As further confirmation that the sedimentable, TF-containing procoagulant material in ARDS edema fluid is composed of MPs, we used a MP capture activity assay to quantitate MPs. ARDS patients have higher concentrations of lipid-containing MPs compared with patients with hydrostatic edema.

Although there are several potential sources of intra-alveolar MPs, including macrophages, platelets, and alveolar epithelial cells, our data suggest that the alveolar epithelium is one potential source of MPs. This finding builds on our previous work demonstrating that the alveolar epithelium upregulates TF in response to inflammation (5), making the alveolar epithelium a likely source of TF-containing MPs. In the current study, alveolar epithelial cells stimulated with proinflammatory cytokines released TF-containing procoagulant MPs, confirming that these cells are capable of releasing MPs when activated. In pulmonary edema fluid, we measured a marker of type I alveolar epithelial cell injury to determine if a portion of the TF MPs originate from the alveolar epithelium. RAGE is reported to be more elevated in the air space in ARDS compared with hydrostatic edema (21). In that study, RAGE measurements were made in pulmonary edema fluid without any centrifugation to remove MPs. In our study, although there was no difference in soluble RAGE between hydrostatic edema and ARDS (data not shown), there was a difference in RAGE levels in MPs between these two groups suggesting that higher RAGE in ARDS MPs was not simply a result of higher total RAGE in the alveolar compartment. It is possible that the differences measured in the previous work by Uchida et al. (21) may have been the result of MP-associated RAGE. Although there are clearly other potential sources of MPs in the alveolar compartment, the finding of elevated RAGE in MPs from patients with ARDS compared with those with hydrostatic edema in the absence of differences in soluble RAGE is evidence that a portion of edema fluid MPs may originate from the alveolar epithelium.

Together, these findings provide the first comprehensive characterization of intra-alveolar procoagulant MPs in patients with ARDS and hydrostatic pulmonary edema. These observations may have important implications for the pathogenesis of ALI. Others have shown that MPs in the circulation are important for amplification of the coagulation cascade and are critical for clot propagation (6, 7). Our data indicate that intra-alveolar MPs contain high levels of TF, are highly procoagulant, and thus are likely to contribute to intra-alveolar fibrin formation, a critical pathogenic feature of ALI. Although there are several potential sources of MPs in the lung (macrophages, lymphocytes, platelets), we have identified the alveolar epithelium as one potential source of intra-alveolar MPs in ALI/ARDS. This finding is novel and may be critically important in understanding the mechanisms of intra-alveolar fibrin deposition. The formation of procoagulant alveolar MPs represents a novel target for therapies aimed at modulating intra-alveolar fibrin deposition. Although the functional significance of intra-alveolar MPs is yet to be determined, our data suggest a potential impact on mortality. These findings are critical first step to understanding the role of intra-alveolar microparticles in modulating fibrin deposition in the lung and to identifying potential new targets for future therapies for ALI/ARDS.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


