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ASSOCIATION FOR ACADEMIC SURGERY:

Endothelial Micro particles Induce Inflammation in Acute Lung Injury

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Abstract

Background—Previously, we have shown that endothelial microparticles (EMPs) injected into mice induce acute lung injury (ALI) [1]. In this study, we hypothesize that EMPs induce ALI by initiating cytokine release in the lung, leading to recruitment and activation of neutrophils.

Materials and methods—C57BL/6J male mice (8–10 wk old) were intravenously injected with EMPs (200,000/mL), LPS (2 mg/kg), or both. Bronchoalveolar lavage (BAL) and serum levels of IL-1 β and TNF- α were analyzed by enzyme-linked immunoassay (ELISA). Morphometric analysis was performed on H and E stained lung sections. Myeloperoxidase (MPO) levels were determined *via* an enzymatic assay and immunofluorescence of stained sections.

Results—EMPs led to significantly increased pulmonary and systemic IL-1 β and TNF- α levels, which correlated with increased neutrophil recruitment to the lung. MPO levels in the lungs were increased significantly following injection of EMPs or LPS, compared to PBS. In mice treated with EMPs and LPS either simultaneously or successively, the cytokine and MPO levels were significantly increased over that of either treatment alone.

Conclusion—EMPs contribute to lung injury through the initiation of a cytokine cascade that increases recruitment of neutrophils and subsequent release of MPO. Furthermore, treatment of mice with both EMPs and LPS induced greater lung injury than either treatment alone, suggesting that EMPs prime the lung for increased injury by other pathogens. Therapies aimed at reducing or blocking EMPs may be a useful strategy for attenuating lung injury.

Keywords

endothelial microparticles; lung injury; IL-1 β ; TNF- α ; myeloperoxidase

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INTRODUCTION

Microparticles (MPs) are submicron vesicles that are released from activated or apoptotic cells, and elevated levels have been correlated with several human disease states such as sickle cell disease, diabetes, and atherosclerosis [2–9]. Recently, tissue factor-positive microparticles (TF-MPs) were shown to be elevated in the bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome (ARDS) [10]. This study implied that TF-MPs are released by activated alveolar epithelium, but also suggests that microparticles may be distributed to the lung and traverse the respiratory barrier. Endothelial microparticles (EMPs) are a subgroup of MPs that are shed from the endothelium in response to a variety of stimuli, including lipopolysaccharide (LPS), autoantibodies, and cytokines such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) [11]. EMPs have been shown to be significantly increased in human disease states in comparison to the other subgroups of microparticles, which include erythrocyte, white blood cell, and platelet MPs [2–9]. Additionally, EMPs have been shown to alter endothelial function in many of these pathologies, implying a role as an injurious agent. Previously, we have shown that EMPs contribute to acute lung injury in a murine model. This conclusion was based on multiple measures of histologic parameters, lung permeability indices, and dysregulation of endothelial nitric oxide formation and release [1].

While several published studies exist quantifying EMPs in human disease and suggesting that they would serve as a worthy biomarker, the mechanisms by which EMPs initiate and propagate disease are still not well understood. In an attempt to further characterize the role of EMPs in lung injury, we investigated their effects on the pulmonary and systemic inflammatory cascade. Cytokines IL1- β and TNF- α have been studied as predictors of survival in AKDS [12]. While their release may be from multiple cell types (i.e., epithelium, monocytes, and macrophages), neutrophils are a large source of these cytokines. As a measure of neutrophil activation in this setting, it implicates neutrophils and serves as a source of further oxidative injury in acute lung injury (ALI).

In human disease with associated ALI/ARDS, there are often multiple pathogenic steps. To explore the possibility that EMPs “prime” the lung for more extensive injury by other pathogens, we explored their effect on cytokine and myeloperoxidase production in a two-hit model of injury using EMPs and lipopolysaccharide (LPS). We reasoned that if EMPs set up the lung for increased damage, the possibility is raised that therapies aimed at preventing release or reducing EMP binding in the lung could play a protective role in reducing the lung injury that accompanies several human disease states.

MATERIALS AND METHODS

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin.

EMPs were generated using previously published protocols [1, 11, 13]. Briefly, human umbilical vein endothelial cells (HUVEC; Clonetics, Walkersville, MD) at passage 3–4 were grown to confluence, then stimulated with plasminogen activation inhibitor-1 (PAI-1, 10

ng/mL; American Diagnostica Inc., Stamford, CT). The supernatant was collected 3 h after stimulation and underwent serial centrifugation and suspension in phosphate buffered saline (PBS). Flow cytometric analysis was used to quantify EMPs based on CD31-positive events occurring in a size gate less than 3 μm in diameter.

For negative controls, we injected mice with inert particles sized 0.7–2.2 μm (Spherotech, Libertyville, IL) and phosphatidylcholine (PC) liposomes produced in our lab by extrusion using published protocols from the manufacturer (Avanti Polar Lipids, Inc., Alabaster, AL). We administered a total of 100 μL of PC liposomes at a concentration of 20 $\mu\text{g}/\mu\text{L}$ after 1 μm filtration.

C57BL/6J mice (Jackson Labs, Bar Harbor, ME), age 8–10 weeks, were randomly separated into five experimental groups. Mice were intravenously injected with filtered PBS, EMPs (200,000/mL estimated blood volume), LPS (2 mg/kg), or EMPs and LPS simultaneously or 6 h apart (200,000/mL estimated blood volume and 2 mg/kg, respectively). Following sacrifice at 24 h, bronchoalveolar lavage (BAL) fluid, serum, and lung tissue were harvested for analysis, as outlined below.

Quantification of IL-1 β and TNF- α by Enzyme-Linked Immunoassay (ELISA)

BAL and serum from the treatment groups were analyzed for changes in IL-1 β and TNF- α . Prior to harvesting lung tissue, anesthetized mice underwent intratracheal lavage with 0.2 mL of filtered PBS three times. Blood collection was performed *via* intracardiac aspiration. Sample processing and ELISA quantification was performed per manufacturer's instructions (R and D Systems, Minneapolis, MN). Briefly, BAL fluid was centrifuged at 10,000 rpm for 10 min, and supernatant was used immediately for ELISA quantification. Blood samples were allowed to clot for 2 h at room temperature prior to centrifugation for 20 min at 2000 g. Serum was assayed immediately in duplicate, and analyzed against a standard curve.

Histology

Prior to harvesting lung tissue for histologic examination, the lungs were flushed with a total of 3 mL of 1% PBS *via* cannulation of the right ventricle. The left lung was then harvested and fixed in 10% zinc formalin prior to paraffin embedding. Slides were stained for hematoxylin and eosin (H and E). Morphometric analysis of H and E slides was performed by an independent pathologist in a similar fashion to our previously published scale, and used the following parameters: polymorphonuclear cell (PMN) count per 100 periaarteriolar cells, edema (0 = none, 1 = moderate, 2 = severe), and red blood cell (RBC) count (0 = none, 1 = compromise of endothelium-alveolar barrier, 2 = diffuse hemorrhage) [1].

Myeloperoxidase Assay

Myeloperoxidase (MPO) activity in lung tissue was determined by methods previously reported [14]. Briefly, right lung tissue was harvested from each mouse at 24 h post-injection, weighed, and immediately frozen in liquid nitrogen. Frozen lung tissue was homogenized in 50 mM potassium phosphate buffer (pH 6.0), centrifuged at 10,000 rpm for 15 min, and the pellet was resuspended in 50 mM potassium phosphate buffer with 50 mM hexadecyltrimethylammonium bromide (HTAB; Sigma-Aldrich, St. Louis, MO). Following

three cycles of snap freezing in liquid nitrogen, thawing, and sonication for 20 s, samples were centrifuged at 10,000 rpm for 10 min. Aliquots of supernatant were added to potassium phosphate buffer containing 0.167 mg/mL o-dianisidine dihydrochloride (Sigma-Aldrich, St. Louis, MO) and 0.0005% hydrogen peroxide. Absorbance was read at 460 nm at 30-s intervals for 2 min.

Immunofluorescence Staining of Lung Tissue

Lungs from the experimental groups were inflated with 10% zinc formalin *ex vivo*, fixed for 48 h, embedded in paraffin, and cut into 5 μ m sections. Slides were deparaffinized and rehydrated in sequential washings of xylene and decreasing alcohol concentrations before antigen unmasking by boiling in 10 mM sodium citrate (pH 6.0) for 20 min, then blocking with 5% goat serum for 20 min. Slides were then incubated with polyclonal MPO antibody (Abeam Inc., Cambridge, MA) at room temperature for 1 h, and fluorescent goat anti-rabbit secondary antibody (Alexa Fluor 633; Invitrogen Corp., Carlsbad, CA) for 1 h at room temperature. Lung sections were incubated with secondary antibody as a negative control. Slides were mounted with 4',6-di-amidino-2-phenylindole (DAPI) fluorescent mounting medium (DAKO North America, Carpinteria, CA) to visualize the nucleic acid background. Confocal microscopy was used to obtain images.

Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD), and comparison between groups was performed with ANOVA test. Significance was determined as $P < 0.05$.

RESULTS

Cytokine Release

TNF- α and IL-1 β are early-response cytokines that are necessary for both initiation and propagation of inflammatory responses. To determine the effect of EMPs on the release of these cytokines, BAL and serum were assayed from the experimental and control groups.

Figure 1A demonstrates the IL-1 β levels in BAL. EMP treatment significantly increased the level of IL-1 β in BAL compared to a PBS-treated control (41.86 ± 0.32 pg/mL *versus* 31.29 ± 0.33 pg/mL, $n = 3$ per group, $P < 0.05$). EMPs caused an increase in IL-1 β in the BAL that was nearly equivalent to that induced by LPS alone (41.86 ± 0.32 pg/mL *versus* 42.36 ± 2.04 pg/mL, $n = 3$ per group, $P = \text{NS}$). Simultaneous administration of EMPs and LPS increased IL-1 β levels in the BAL to a level that was greater than either experimental treatment alone (49.35 ± 1.23 pg/mL, $n = 3$, $P < 0.05$ compared with EMP and LPS groups). Injection of LPS 6 h following EMP administration produced a significant increase in BAL IL-1 β level compared with either treatment alone or in combination (69.16 ± 7.85 pg/mL, $n = 3$, $P < 0.05$ compared with other experimental groups). Injection of beads or PC liposomes did not increase IL-1 β levels over PBS treatment (33.42 ± 0.58 pg/mL and 34.31 ± 1.37 pg/mL, respectively, *versus* 31.29 ± 0.33 pg/mL, $n = 3$ per group, $P = \text{NS}$; data not shown in this or subsequent graphs.).

EMP treatment increased serum IL-1 β levels compared with PBS-treated control mice, as shown in Fig. 1B (35.34 ± 0.63 pg/mL *versus* 26.57 ± 1.05 pg/mL, $n = 3$ per group, $P = 0.05$). However, injection of EMPs alone did not increase serum IL-1 β to the same level as induced by LPS (35.34 ± 0.63 pg/mL *versus* 43.66 ± 1.05 pg/mL, $n = 3$ per group, $P = 0.05$). Mice treated with EMPs and LPS at the same time expressed higher levels of serum IL-1 β than either treatment alone (47.52 ± 0.57 , $n = 3$, $P = 0.05$ compared with EMP and LPS groups). Sequential administration of EMPs followed by LPS led to a significantly higher level of IL-1 β in serum than simultaneous injection of the two agents (130.64 ± 20.75 pg/mL *versus* 47.52 ± 0.57 pg/mL, $n = 3$, $P = 0.05$). Bead and PC liposome injection did not increase serum IL-1 β level over mice injected with PBS (30.02 ± 0.28 and 30.69 ± 0.94 pg/mL *versus* 26.57 ± 1.05 pg/mL, $n = 3$, $P = \text{NS}$).

TNF- α in BAL fluid and serum followed a similar pattern of expression in response to EMP and/or LPS treatment. As seen in Fig. 2A, EMPs significantly increased BAL TNF- α levels compared with the levels in PBS-treated mice, and was similar in effect to LPS treatment (PBS: 100.30 ± 2.33 pg/mL, EMP: 149.52 ± 12.15 pg/mL, LPS: 142.92 ± 0.22 pg/mL; EMP *versus* PBS, $P = 0.05$; EMP *versus* LPS, $P = \text{NS}$; $n = 3$ per group). EMPs and LPS in combination again increased TNF- α to a greater level of expression than either treatment alone (169.53 ± 0.90 pg/mL, $n = 3$, $P = 0.05$ compared with EMP and LPS alone), and led to a significantly increased level when administered 6 h apart (239.23 ± 46.83 pg/mL, $n = 3$, $P = 0.05$ compared with simultaneous injection). Treatment with beads and PC liposomes did not differ from PBS injection (106.62 ± 6.72 and 102.30 ± 1.51 pg/mL *versus* 100.30 ± 2.33 pg/mL, $n = 3$, $P = \text{NS}$).

Figure 2B demonstrates the effect of EMPs on serum levels of TNF- α , which again were increased compared with PBS treatment, but not to the extent of LPS treatment (PBS: 106.39 ± 0.96 pg/mL, EMP: 120.77 ± 4.60 pg/mL, LPS: 130.57 ± 1.44 pg/mL; $n = 3$ per group; $P = 0.05$ for PBS *versus* EMP and EMP *versus* LPS). Simultaneous administration again demonstrated an increase in TNF- α levels over that of either treatment alone (141.50 ± 5.31 pg/mL, $n = 3$, $P = 0.05$ compared with EMP and LPS groups). Injection with EMPs followed in 6 h by LPS demonstrated significant increases in serum TNF- α levels *versus* co-administration of the two agents (438.88 ± 83.27 pg/mL *versus* 141.50 ± 5.31 pg/mL, $n = 3$, $P = 0.05$). Bead and PC liposome treatment did not induce significant changes in serum TNF- α level compared with PBS (104.68 ± 5.80 and 109.06 ± 1.82 pg/mL *versus* 106.39 ± 0.96 pg/mL, $n = 3$, $P = \text{NS}$).

Neutrophil Recruitment

Morphometric analysis of H and E stained lung tissue from the treatment groups is shown in Table 1. Representative lung sections used for morphometric analysis are shown in Figs. 3 and 4. An increase in perivascular aggregation of neutrophils can be seen in the EMP treatment group alone and followed in 6 h by LPS, but similar increases were not observed in the LPS alone or EMP/LPS co-treatment groups. Perivascular edema was increased in all four experimental groups compared with the PBS control. Compromise of the endothelial/alveolar barrier as noted by intra-alveolar hemorrhage (RBCs) was increased over baseline only in the LPS treatment group. The number of neutrophils in the pleura and alveolar space,

termed “pleuritis,” was significantly increased in all four treatment groups compared with PBS control lungs (Fig. 4).

Myeloperoxidase Levels

We next investigated whether the recruitment of neutrophils to the lung translated to activation and subsequent increase in MPO levels in the lung tissue. Lung tissue from each treatment group was analyzed for MPO by the o-dianisidine dihydrochloride oxidation assay and by immunofluorescence staining.

As seen in Fig. 5A, MPO activity in the lungs was increased following injection of EMPs or LPS alone compared to PBS (PBS: 0.08 ± 0.01 , EMP: 0.22 ± 0.14 , LPS: 0.98 ± 0.10 activity units/g lung tissue; $n = 3$ per group; $P = 0.05$ for intergroup comparisons). In mice treated with EMPs and LPS simultaneously, MPO activity was significantly increased over that of either treatment alone (EMP/LPS: 2.01 ± 0.12 , EMP: 0.22 ± 0.12 , LPS: 0.98 ± 0.10 activity units/g lung tissue; $n = 3$ per group, $P = 0.05$ for intergroup comparisons). Injection of EMPs followed in 6 h by LPS led to significantly increased MPO activity over that of co-administration of the two agents (3.25 ± 0.22 versus 2.01 ± 0.12 activity units/g lung tissue, $n = 3$, $P = 0.05$). PC liposome and bead injection did not increase MPO activity over that of PBS (0.05 ± 0.01 and 0.05 ± 0.02 versus 0.08 ± 0.01 activity units/g lung tissue, $n = 3$, $P = \text{NS}$).

Immunofluorescence staining of lung tissue confirmed the results of the MPO assay. As seen in Fig. 5B, while both EMPs and LPS each individually increased MPO staining in the lung microvasculature over PBS, co-injection of EMPs and LPS significantly increased accumulation of MPO in the lung compared with either treatment alone. LPS injected 6 h after EMP treatment led to a significant increase in MPO staining in the microvasculature over that of simultaneous injection.

DISCUSSION

The data presented here offer new insight into the mechanisms by which EMPs contribute to acute lung injury (ALI), and specifically focus on inflammatory cytokines and myeloperoxidase release. Previously, these mechanistic steps have not been described in our model of lung injury. In humans with acute respiratory distress syndrome (ARDS), an increase in the level of procoagulant tissue factor-positive microparticles (TF-MPs) in the alveolar space has been recently described. This study suggests that alveolar epithelium activated by cytokines may lead to increased production of TF-MPs, or that microparticle translocation across the respiratory barrier may be an important event in ALI [10]. Our morphometric data indicate that EMPs recruit neutrophils specifically and induce acute lung injury when given intravenously compared to LPS alone. The injury induced by EMPs is augmented by a second hit of LPS, which correlates well with the clinical scenario of ALI accompanied by a systemic inflammatory response. This is the first demonstration of deferential cytokine release caused by EMPs, implicating specificity for lung injury. Additionally, this report highlights the importance of EMPs as a priming step in a model consistent with multifactorial disease.

Levels of IL-1 β and TNF- α in BAL fluid of patients with ARDS correlate with survival [12]. The release of IL-1 β and TNF- α is essential to the initiation and promotion of a cascade of events leading to recognition and ultimately tissue repair. These cytokines also prime neutrophils, leading to enhanced oxidative burst, release of reactive oxygen metabolites, degranulation and release of a number of proteolytic enzymes [15–19]. As demonstrated in our ELISA assays, EMPs increase pulmonary levels of IL-1 β and TNF- α . LPS is a nonspecific inducer of systemic inflammation; by comparison, EMPs have a relatively greater effect in the lung than the periphery with regard to cytokine release. This targeted inflammatory response with EMP treatment correlates well with our previous observations that EMPs are preferentially distributed to the lung (data not shown). This suggests that EMPs work with some specificity in the lung tissue to initiate a cascade of events that ultimately leads to further end-organ damage.

Elevations in cytokine production parallel increased neutrophil recruitment and ALI, as demonstrated by morphometric analysis of lungs from treated mice. Myeloperoxidase is one of the principal enzymes released by activated neutrophils, and is a large source of oxidative injury in the lung. While our previous studies have shown similar increases in myeloperoxidase activity in EMP-treated animals, the work presented here demonstrates an augmentation of this effect by LPS. Unlike our findings with BAL cytokine release, EMP-induced MPO activity in the lung tissue appeared to be less than that induced by LPS. However, the cumulative effect on MPO release in the lung from these two agents given together, and significant increase in MPO levels when LPS is given as a “second hit” 6 h after EMP administration, is relevant clinically in multifactorial sepsis. Interestingly, EMPs appear to specifically recruit neutrophils to the lung when given alone or as a priming agent before LPS administration. One possible explanation may be that the systemic inflammatory response induced by LPS serves as a signal to prevent the specific accumulation of neutrophils in the lung. Regardless, EMPs and LPS appear to be synergistic inducers of lung MPO activity, an effect which is significantly magnified when EMPs and LPS are successively administered in a fashion which would mimic human pathology.

Taken together, these data offer new evidence that EMPs are significant contributors to ALI *via* inflammatory cytokines, resultant neutrophil recruitment, and ultimately increases in MPO levels in the lung tissue. Lung injury is a component of several human disease states in which EMP levels are significantly increased. Our previous observation of EMP sequestration in the lung and its ability to initiate inflammatory cascades make a compelling argument that targeting EMPs may hold therapeutic value for protecting the lung in a variety of diseases.

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FIG. 1.

Effects of EMPs on BAL (A) and serum (B) IL-1 β after treatment with phosphate-buffered saline (PBS), endothelial microparticles (EMP), lipopolysaccharide (LPS), and simultaneous (EL), or sequential (E/L) treatment with EMPs and LPS. Error bars represent standard deviation (SD); * $P < 0.05$; $n = 3$ per group.

FIG. 2.

Effects of EMPs on BAL (A) and serum (B) TNF- α after treatment with phosphate-buffered saline (PBS), endothelial microparticles (EMP), lipopolysaccharide (LPS), and simultaneous (EL), or successive (E/L) treatment with EMPs and LPS (EMP/LPS). Error bars represent standard deviation (SD); * $P < 0.05$; $n = 3$ per group.

FIG. 3.

H and E stained lung sections (20× magnification) from mice treated with PBS (A), EMPs (B), LPS (C), and simultaneous (EL), or sequential (E/L) treatment with EMPs plus LPS (D) and (E), respectively. Perivascular edema is notable in the four experimental treatment groups compared with PBS control (black arrow heads). (Color version of figure is available online.)

FIG. 4.

Representative H and E stained sections of lung tissue (20× magnification) from mice treated with PBS (A), EMPs (B), LPS (C), and simultaneous (EL), or successive (E/L) injection of EMPs plus LPS (D) and (E), respectively, demonstrating increasing degrees of pleuritis (black arrow heads), edema, and alveolar hemorrhage. (Color version of figure is available online.)

FIG. 5.

Myeloperoxidase activity (A) and immunofluorescent staining (B) in lung tissue. Increasing amounts of MPO staining (B; white arrows) can be seen in the vascular wall and parenchyma after injection with EMPs, LPS, co-administration of EMPs and LPS (EL), and EMPs followed in 6 h by LPS (E/L) compared with PBS. (Color version of figure is available online.)

TABLE 1**Morphometric Analysis of H and E Stained Lung Tissue**

	PMNs/100 periarteriolar cells	Edema (0–2)	Hemorrhage (0–2)
PBS	5 ± 4	0	0.33
EMP	11 ± 10	0.33	0.33
LPS	2 ± 1	1	0.67
EL	1 ± 1	1.67	0.33
E/L	19 ± 3	2	0

An increase in perivascular PMNs is noted in the EMP treatment group. Increasing edema is seen in the EMP and LPS treated mice. Mice treated with LPS 6 h after EMP treatment (E/L) show significant increases in perivascular PMNs and edema compared with co-administration of EMPs and LPS (EL) and either treatment alone.