Administration of microparticles from blood of the lipopolysaccharide-treated rats serves to induce pathologic changes of acute respiratory distress syndrome

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Abstract

This study was conducted to investigate the effect of intratracheal and intravenous administration of microparticles (MPs) on developing acute respiratory distress syndrome (ARDS). The blood MPs from lipopolysaccharide-treated rats were collected and examined by transmission electron microscopy (TEM). Cellular source of the MPs was identified by fluorescent-labeled antibodies after the circulating MPs were delivered to naïve rats. Levels of myeloperoxidase (MPO), tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-10 productions in bronchoalveolar lavage fluid (BALF) and plasma were determined 24 h after the rats received intratracheal and intravenous administration of the MPs. Histopathologic examination of lungs was performed by light microscope. A TEM image of MPs showed spherical particles at a variable diameter from 0.1 to 0.5 μ m. Endothelial- and leukocyte-derived vesicles were abundant in the investigated samples. Treatment with MPs may lead to significant increases in MPO, TNF- α , IL-1 β , and IL-10 productions in BALF and plasma of the rats (all $P < 0.001$). Morphological observation indicated that alveolar structures were destroyed with a large amount of neutrophil infiltration in the lungs of the MP-treated rats. Perivascular and/or intra-alveolar hemorrhage were serious and hyaline membrane formed in the alveoli. Intratracheal and intravenous approaches to delivery of the circulating MPs to naïve recipient rats may induce ARDS. This presents an inducer of the onset of ARDS and provides potential therapeutic targets for attenuating lung injury.

Keywords: Microparticles, acute respiratory distress syndrome, myeloperoxidase, cytokines, pulmonary hyaline membranes

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Introduction

Acute respiratory distress syndrome (ARDS) is a life-threatening lung disease caused by a variety of direct and indirect insults. It is characterized by inflammation of the lung parenchyma leading to impaired gas exchange with concomitant systemic release of inflammatory mediators causing inflammation and hypoxemia.¹ Pathogenesis of ARDS has not been fully elucidated, but the disease as the most severe form of acute lung injury is often associated with increased production of cytokines and inflammatory mediators from circulating cells, local epithelial and endothelial cells.^{2,3} Despite the well-documented benefit of protective ventilatory strategies and increasing knowledge of the etiological mechanisms of ARDS, mortality rates remain relatively stagnant around 40%.⁴

Microparticles (MPs) are tiny cell-derived, intact vesicles which can be released by activated or apoptotic cells.^{5,6}

It has been reported that concentration of MPs in ARDS patients is elevated,⁷ and the circulating MPs intravenously injected to mice may induce ARDS by initiating cytokine release in the lungs, leading to recruitment and activation of neutrophils.⁸ Moreover, there has been growing awareness that distinct populations of MPs in both the vascular and alveolar compartments appear in ARDS animal models and ARDS patients, which may serve as diagnostic and prognostic biomarkers.⁹

In effort to understand a crucial role of circulating MPs in developing ARDS, the MPs were first harvested from an animal model of the lipopolysaccharide (LPS)-induced ARDS. The levels of inflammatory mediators in bronchoalveolar lavage fluid (BALF) and plasma were then examined by intratracheal and intravenous delivery of the particles to rats. Morphological changes of lung tissues from the MPs-treated animals were observed as well. Our results indicate that the circulating MPs are an inducer of ARDS with

potential targets aimed at reducing their content for treatment of ARDS.

Materials and methods

Animals and reagents

Specific pathogen-free, male Wistar rats (6–8 weeks) weighing 200 g were purchased from Academy of Military Medical Sciences, Experimental Animal Research Centre. The rats were housed in an environmentally controlled animal facility of our hospital with relative humidity of 40–70% at 18–28°C for the duration of the experiments. Biological samples and specimens from experimental rats were harvested under general anesthesia. All procedures were reviewed and approved by Hospital Research Review Committee.

LPS and polystyrene particles (PP) were purchased from Sigma and SpheroTech (USA), respectively. Annexin V-APC Apoptosis detection kit was purchased from Nanjing KGI Biotechnology Development Co., Ltd. (Nanjing, China). Fluorescent-conjugated antibodies for differentiation analysis, including CD61, CD45, and CD54 were purchased from BD Bioscience, Inc. (San Diego, CA USA). Enzyme-linked immunosorbent assay (ELISA) kits for myeloperoxidase (MPO) and cytokine research were purchased from Invitrogen Life Technologies (Carlsbad, CA USA). Bicinchoninic acid assay (BCA) Kit was obtained from Beijing Pulilai Technology Co., Ltd. (Beijing, China). All reagents are commercially available.

Preparation of MPs

Eighteen male rats were randomly divided into three groups of six and received surgical procedures. Briefly, the tracheas of the animals were shown up in minimally invasive approach after they were anesthetized by intraperitoneal injection of 2% sodium pentobarbital (50 mg/kg). Two hundred microliters of normal saline (NS) and LPS (10 mg/kg dissolved in 200 μ l physiological saline) were delivered into the trachea by needles. The rats received no treatment as the control group.

Blood samples of the rats were collected in tubes containing citrate anticoagulant 24 h after the treatments since cell-derived vesicles including MPs are abundantly present in blood. MPs were obtained using three centrifugation steps: (1) Blood samples in the tubes were centrifuged (1500g $\times$ 20 min) at 4°C to collect the cell pellets. Then, 1.0 ml of plasma from each tube was transferred into separate sterile Eppendorf tubes (1.5 ml); (2) platelet-poor plasma was collected by centrifuge (13,000g $\times$ 2 min) at 4°C, and the supernatants were transferred into new sterile tubes, avoiding the last 100 μ l at the base of the centrifuged tubes; (3) a microparticle pellet from the platelet-poor plasma was again centrifuged (21,000g $\times$ 60 min) at 4°C. Subsequently, the supernatant was discarded, and the microparticle pellet was reconstituted in Annexin V buffer. Buffers were sterile-filtered with 0.22 μ m filters in this procedure.

Detection of MPs

A transmission electron microscopy (TEM) utilizes energetic electrons to provide morphologic and compositional information on samples. It is capable of imaging at a significantly higher resolution than light microscopes, owing to the small de Broglie wavelength of electrons. Imaging systems in a TEM consist of a phosphor screen, which may be made of fine (a maximum potential magnification of 1 nm) particles for direct observation. Three rats in each group were randomly selected for detection of blood MPs in a TEM. Copper wire mesh was prepared for this procedure. Briefly, 20 μ l of MPs suspension was placed on copper wire mesh for 5 min, and excess liquid on the mesh was absorbed using filters. Then, 20 μ l of phosphotungstic acid was added and incubated for 10 min. MPs were examined by TEM after the liquid on the mesh was removed again using filters.

In this study, we focus on the relatively high throughput detection of vesicles in suspension by flow cytometry using fluorescent-conjugated antibodies, including CD61 (a membrane protein present on platelets), CD45 (a type I transmembrane protein present on leukocytes), and CD54 (a type I transmembrane protein present on endothelial cells), followed by staining with APC Annexin V Apoptosis Detection Kit. Briefly, forward and side scatter light double logarithmic scales were plotted in a single dimension (FSC-H $\times$ SSC-H). The regions on these plots can be sequentially separated based on fluorescence intensity, by creating a series of subset extractions in reference to deionized water detection. With multiplex flow cytometry analysis, circulating MPs from blood were defined by their size, which ranges between 0.1 and 0.9 μ m. Cellular sources of the MPs were identified by measurement of fluorescence intensity of the labeled antibodies.

Approach of MP-induced ARDS

Thirty-six naïve recipient rats were divided into six groups and were treated with either intratracheal (T) or intravenous (V) injection of NS, PP, and MP, respectively. Pathologic changes of ARDS were examined in plasma, BALF, and lung tissues from the rats that received treatments of T-NS and V-NS, T-PP and V-PP (3.5 $\times$ 10⁷/kg), and T-MP and V-MP (3.5 $\times$ 10⁷/kg) 24 h after the initial treatment in this study. Concentration of the particles applied for this experiment was selected in reference to the study.¹⁰ Additionally, the dose of the MPs selected for each animal also referred to an average concentration from individual blood samples of the diseased animals. PP was prepared in an appropriate concentration for FACS examination. Briefly, 100 μ l of PP were diluted in 900 μ l of phosphate-buffered saline (PBS) and mixed in 1.5-ml tube. The supernatant in the tube was discarded with centrifuge (25,000g $\times$ 15 min). The PP remaining in the tube was washed with PBS. A final concentration of PP used was the same in dosage and volume as MP.

Experimental animals were sacrificed, and the left lung of the rats was lavaged twice with 3.0 ml of PBS after the right main bronchus was ligated. Recovered BALF of 87–97% was collected, and the BALF supernatant was obtained

with centrifuge ($3000g \times 15$ min) at 4°C . Blood from rats was collected, and blood plasma was obtained with centrifuge ($3000g \times 15$ min) at 4°C . All samples were stored at -80°C until the ELISA.

The contents of MPO, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and IL-10 productions in BALF and plasma were determined in duplicates using antibodies against the murine MPO and special cytokines according to the manufacturer's directions. Briefly, 100 μl of horseradish peroxidase-conjugated antibodies were added to each well containing 10 μl of sample in 40 μl of PBS in a microtiter plate. The plate was incubated for 60 min at 37°C . The solution in each well was discarded and washed five times with PBS. Then, 50 μl of the substrate solution was added in each well and incubated for 15 min at room temperature. The reaction was stopped by adding 50 μl of 4 M sulfuric acid. The OD values were read in a microtiter autoreader at 450 nm. The protein concentrations ($\mu\text{g}/\text{ml}$) in the supernatant of the BALF and the plasma were examined by BCA. Absorbance of total protein from each group was measured at 562 nm.

Histopathologic examination

Experimental animals were sacrificed by opening chest. The lower lobe of right lung was surgically removed, weighing 100 mg individually. The tissues were fixed with

formaldehyde (10%) for 24 h, and then embedded in paraffin before cutting into 5- μm sections. The sections were stained with hematoxylin and eosin (H&E) solution. Slides were observed under a light microscope at a magnification of 400 for morphological analysis.

Statistic analyses

Values were expressed mean $\pm$ standard deviation (SD) on the results of MPO and cytokine contents. Statistical analyses were performed using Statistical Package for the Social Science (SPSS, version 17.0). Student's paired *t*-test was used to compare measurements of individual groups. Significance was accepted at $P < 0.05$.

Results

Features and distribution of MPs

Features of MPs from blood samples of control, NS- and LPS-treated rats were observed in a TEM image, and the result is shown in Figure 1(a). Scanning electron micrographs of the MPs in the sample showed spherical particles of the particles with variable diameters of 0.1–0.5 μm in size. Those of MPs in the image were captured at 20,000 $\times$ magnification. In contrast, population distribution of MPs in all of three groups was visually uneven in the field of same

Figure 1 Features of microparticles. Rats were grouped as control, NS, and LPS-induced ARDS, respectively. Blood samples from the animals were collected 24 h after the treatments. TEM images (a) of MPs showed spherical particles at a variable diameter of 0.1–0.5 μm . Image was captured at a magnification of 20,000. Population distribution (b) of circulating MPs in blood from animal groups (control, NS, and ARDS) was analyzed by fluorescent-labeled antibodies of CD61 (left), CD45 (middle), and CD54 (right), and then stained with APC Annexin V Apoptosis Detection Kit. Significant increases in a plasmatic level of leukocyte (CD45)- and endothelial (CD54)-derived microparticles were observed in the ARDS group as compared to others. Measurements were expressed as mean $\pm$ SD ($n = 6$). NS: normal saline; ARDS: acute respiratory distress syndrome

Figure 2 Level of myeloperoxidase in BALF and plasma. Contents of MPO production in BALF and plasma were assessed in rats that received intratracheal and intravenous injection of MPs. Concentration of MPO in the MP-treated samples was significantly increased as compared to other treatments. Measurements were expressed as mean $\pm$ SD ($n = 6$).

BALF: bronchoalveolar lavage fluid; MPO: myeloperoxidase; NS: normal saline; PP: polystyrene particles; MP: microparticle

size, and the number of MPs in the LPS-treated rats was higher than these in other groups.

A distribution of MP population from blood sample of rats that received treatments (NS, PP, and MP) was examined using fluorescent-labeled antibodies of CD61, CD45, and CD54, and then stained with APC Annexin V Apoptosis Detection Kit. The results are shown in Figure 1(b). CMP counts ($10^2/\mu\text{l}$) from blood samples labeled with CD61+, CD45+, and CD54+ showed as 4.90 ± 1.0 , 0.12 ± 0.06 , and 0.04 ± 0.01 in control, 5.10 ± 1.40 , 0.14 ± 0.05 , and 0.05 ± 0.02 in NS, and 5.0 ± 1.2 , 0.21 ± 0.08 , and 0.15 ± 0.04 in ARDS, respectively. In contrast, the concentrations of the MPs labeled with CD45 and CD54 were significantly different with 1.5- and 3.0-fold increases over the NS-treated rats. There were statistic differences detected in the plasmatic levels of endothelial- and leukocyte-derived MPs between ARDS and other groups (both $P < 0.05$; $n = 6$).

Level of MPO

Content of MPO in BALF and plasma from rats that received intratracheal and intravenous administration of MPs was assessed, and the results are shown in Figure 2. An average level (pg/ml) of MPO in BALF and plasma displayed as 479.14 ± 31.54 and 578.32 ± 76.23 in T-NS, 502.32 ± 66.2 and 592.5 ± 85.04 in T-PP, 2260.94 ± 122.67 and 1793.71 ± 68.08 in T-MP, 459.59 ± 42.46 and 481.63 ± 43.58 in V-NS, 454.0 ± 45.52 and 494.81 ± 55.70 in V-PP, and 2059.4 ± 155.14 and 1860.96 ± 59.70 in V-MP, respectively. In contrast, similar changes in the MPO levels were seen in the rats treated with T-NS, T-PP, V-NS, and V-PP. However, the MPO contents in BALF and plasma from the MP-treated rats were significantly different with at least a 4.5- and 3.1-fold increase as compared to other treatments (all $P < 0.001$, $n = 6$).

Content of cytokines

Levels of cytokines in BALF and plasma from rats that received treatments were assessed, and the results are shown in Figure 3. An average concentration (pg/ml) of TNF- α production in BALF and plasma displayed as 389.15 ± 57.61 and 385.86 ± 33.69 in T-NS, 396.93 ± 54.14 and 397.28 ± 38.75 in T-PP, 1354.66 ± 61.08 and 1165.66 ± 67.98 in T-MP, 392.62 ± 33.23 and 371.98 ± 42.14 in V-NS, 387.21 ± 33.84 and 380.69 ± 29.33 in V-PP, and 1252.66 ± 53.49 and 1044.68 ± 77.64 in V-MP, respectively. Content of the cytokine in BALF and plasma was significantly increased in the MP-treated rats with 3.4- and 3.2-fold changes as compared to others ($P < 0.001$, $n = 6$).

An average content (pg/ml) of IL-1 β in BALF and plasma was shown as 51.12 ± 5.6 and 55.31 ± 8.97 in T-NS, 54.5 ± 10.6 and 49.14 ± 4.53 in T-PP, 183.75 ± 11.63 and 140.2 ± 4.61 in T-MP, 49.49 ± 9.4 and 50.77 ± 5.19 in V-NS, 50.24 ± 5.96 and 52.67 ± 4.02 in V-PP, and 179.9 ± 12.72 and 119 ± 7.12 in V-MP, respectively. Levels of IL-1 β obviously enhanced in the MP-treated rats as compared to others ($P < 0.001$, $n = 6$).

An average level (pg/ml) of IL-10 in BALF and plasma was expressed as 60.09 ± 9.38 and 67.24 ± 9.48 in T-NS, 60.44 ± 8.64 and 66.71 ± 11.5 in T-PP, 216.47 ± 10.94 and 220.79 ± 10.65 in T-MP, 57.86 ± 7.04 and 60.34 ± 6.61 in V-NS, 55.72 ± 7.78 and 63.09 ± 7.64 in V-PP, and 216.73 ± 13.25 and 206.92 ± 11.11 in V-MP, respectively. Treatment with MPs resulted in increased production of IL-10 as compared to other treatments ($P < 0.001$, $n = 6$).

Histopathologic analysis of lungs

Cross-sectional areas of lung specimens from the MP-treated rats were photographed under a light microscopy at a magnification of 400, and images of pathologic examination of the lung tissues are shown in Figure 4. Our results showed the lungs appeared deep purplish red and were

Figure 3 Levels of cytokine productions in BALF and plasma. Concentrations of TNF- α , IL-1 β , and IL-10 productions were determined in BALF (upper row) and plasma (lower row) from the rats that received intratracheal and intravenous administration of MPs. The levels of the cytokines in the samples were significantly raised with a statistical difference as compared to the animals treated without MPs. Measurements were expressed as mean $\pm$ SD ($n=6$). BALF: bronchoalveolar lavage fluid; TNF: tumor necrosis factor; NS: normal saline; PP: polystyrene particles; MP: microparticle; IL: interleukin

Figure 4 Morphological analysis of lung tissues. Histological examination was performed on cross-sectional area of lung specimens from the rats treated with and without MPs under a light microscopy ($\times 400$). Alveolar structures were almost unrecognizable with a lot of neutrophil infiltration within the field of vision. Perivascular and/or intra-alveolar hemorrhage and early hyaline membrane formation were widely observed in the alveoli. (A color version of this figure is available in the online journal.)

NS: normal saline; PP: polystyrene particles; MP: microparticle

liver-like in consistency. Morphologically, the rats that received intratracheal and intravenous injection of either NS or PP showed a normal structure of alveoli with an intact epithelium layer. Those of rats were free of cellular

infiltration with alveolar septa clearly seen in the sections. In contrast, alveolar structures in the T-MP- and V-MP-treated rats were almost unrecognizable with a large amount of neutrophil infiltration in the lung parenchyma

as well as interstitial and alveolar edema. Perivascular and/or intra-alveolar hemorrhage were extremely serious with pulmonary hyaline membrane formed in the alveoli in the morphological observation of the sectional tissues.

Discussion

Features of circulating MPs in the blood samples from control, NS, and the LPS-induced ARDS groups showed spherical particles at a variable diameter of 0.1–0.5 μm in TEM images, indicating that MPs were small membrane bound vesicles circulating in the blood derived from cells that were in contact with the bloodstream such as leukocytes, platelets, and endothelial cells. Although the circulating MPs can be found in the blood of normal animals, a considerable increase in the MPs may be observed in the ARDS group. Because MPs may readily circulate in the vasculature, it is reasonable to postulate that they serve as shuttle modules and signaling transducers not only in their local environment but also at remarkable distance from their site of origin.

In terms of population density and source of the circulating MPs, main classification was performed in proportion of cell-derived MPs from the treated rats. Our results showed that most of the MPs were defined as the endothelial and leukocyte-derived vesicles which showed an increased fluorescence intensity of CD45 and CD54 in the LPS-treated animals. However, there was similar population distribution detected in the platelet-derived MPs in the given samples. These findings not only indicated cellular sources of the blood MPs but also suggested its biological activity based on the fact that cellular microvesicles are fragments of the plasma membrane of parental cell, typically from the surface of blebbing membranes that arise after activation or apoptosis of the cell.^{11–13} Since the sources of the fluorescent vesicles were clarified in the animal model of ARDS, it raised the possibility that appearance of the MPs referred to severity of endothelial injury and inflammatory response in the diseased lungs. It has been known that circulating MPs released by budding of the plasma membrane from these parental cells display a broad spectrum of bioactive substances as veritable vectors for the intercellular exchange of biological signals and transferred part of their components to selected target cells.¹⁴ Such it is likely that the particles exert separable effects involving both the endothelial cell and leukocytes, representing different stages in process of ARDS development.

In order to investigate whether circulating MPs may induce ARDS in naïve recipient rats, an experimental concentration of the MPs was selected according to the finding that MPs are capable of inducing significant lung injury.¹⁰ In this study, the effects of the MP-inducing ARDS were verified by measurements of pro-inflammatory protein mediators in lungs of the MP-treated animals. Concentrations of MPO and cytokines in BALF and plasma were assessed based on the consideration that release of inflammatory mediators would be one of the important features in pathogenesis of ARDS.¹⁵ Two approaches were applied to measure levels of MPO and

cytokines in the biological fluids because their responses normally are compartmentalized in the lungs, and the study of blood specimens provides an incomplete reflection of inflammatory events in the lungs.¹⁶ The data showed that the MPs induced not only high levels of MPO, as well as high levels of TNF- α , IL-1 β , and IL-10 in both samples as compared to other treatments. This suggested that the concentration of the MPs applied to the rats was sufficient to cause releases of the inflammatory mediators in induction of ARDS. Since intratracheal and intravenous administration of the particles showed similar activity in the effect of the mediators released 24 h after treated with the MPs, our postulate was that compartmentalization was gradually lost to some extent during severe inflammatory responses. It has been found that MPO as a potent tissue damage factor from activated neutrophils is associated with aggravation of inflammatory damage in the lungs,¹⁷ and a large variety of inflammatory mediators including TNF- α , IL-1 β , and IL-10 are released in the early phase of ARDS.^{18,19} Our findings, like those seen in patients with ARDS,^{20,21} fully demonstrated the association of ARDS and lung inflammation responses at the early phase of the aggressive lung disease in this animal model. Since rats treated with the blood MPs had an over-production of MPO and cytokines in BALF and plasma samples, it provides important clues to the MPs' role in such pulmonary disease as ARDS and thus may lead to novel therapeutic intervention in the onset of ARDS.

Histopathological changes of lungs showed an acute response in an exudative phase with interstitial and alveolar edema 24 h after the rats were treated with MPs. Accumulation of inflammatory cells and red blood cells in the alveoli were seen with diffuse alveolar damage, as well as hyaline membrane formation, suggesting the morphological abnormalities existed in the early phase of ARDS. Since there were no differences in the pathologic changes of the lung tissues between intratracheal and intravenous injection of the MPs, it led us to conclude that the two approaches used in this study were feasible to develop ARDS. It has been clear that the pathology most commonly associated with ARDS is diffuse alveolar damage which is characterized by a diffuse inflammation of the lung.²² Furthermore, the triggering insult to the lung usually results in an initial release of cytokines and other inflammatory mediators secreted by local epithelial and endothelial cells. In association with the increases in MPO and cytokine productions, it is reasonable to consider that the circulating MPs originated from endothelial cells and leukocytes can be a direct factor contributed to the event of ARDS.

In conclusion, our results not only reveal the main cell sources of the circulating MPs but also demonstrate that intratracheal and intravenous approaches to delivery of the blood vesicles to naïve recipient rats may induce ARDS. Removal of MPs and/or inhibition of MP functions would be a promising strategy for future treatment intervention.

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and review of the manuscript; HL, XM, YG, SC, and XL conducted the experiments; XL supplied critical reagents; HL, XM, and XL wrote the manuscript. HL and XM contributed equally to this work.

CONFLICT OF INTEREST

None declared.

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