Isolation and phenotypic characteristics of microparticles in acute respiratory distress syndrome

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Abstract: Objective: To investigate the alterations of microparticles in acute respiratory distress syndrome (ARDS) in rats. Methods: 18 Wistar male rats were randomly divided into three groups: no intervention, sham (saline control) group and ARDS group (LPS induced). Blood was collected from abdominal aorta and microparticles were extracted through multiple rounds of centrifugation. Particles were analyzed by flow cytometry and transmission electron microscope. Results: The circulating concentration of total microparticles of rats with ARDS induced by lipopolysaccharide (LPS) did not change compared with other two groups. However, ARDS rats expressed higher concentration of leukocyte- and endothelium-derived microparticles in the three groups. Conclusion: Our results indicate that leukocyte and endothelial cell-derived particles may play an important role in ARDS. Thus it is important not only to monitor total microparticle levels but also the phenotypes, which may contribute to the prevention and early treatment of ARDS.

Keywords: Acute respiratory distress syndrome, microparticles, inflammatory mediator

Introduction

Acute respiratory distress syndrome (ARDS), characterized by widespread inflammation of the lungs, leads to severe diseases of the alveolar epithelium. Pulmonary capillary damage, diffuse alveolar capillary permeability, non-cardiogenic pulmonary edema, transparent film formation and lung collapse are the main pathological changes to progressive respiratory distress and refractory hypoxemia clinical features of ARDS. Despite some development in the treatment using mechanical ventilation strategy, ARDS mortality is still high at 30%-50% [1].

The pathogenesis of ARDS has not yet been fully elucidated; it is however known to be mediated by overexpression of inflammatory mediators. Various pulmonary and extra pulmonary injury factors can activate macrophages, neutrophils and release large amounts of pro-inflammatory cytokines, inclusive of tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and anti-inflammatory mediators IL-10, IL-1Rα, forming a waterfalls cascade reaction, eventually leading to lung injury. Inflammation associated cell shedding release large amounts of sub-micron vesicles, known as microparticles (MPs). Many studies have confirmed that particles are assessed in many inflammatory diseases cell activation or apoptosis-sensitive indicators, such as systemic inflammatory response syndrome, meningococcal sepsis, and severe trauma [2-4]. Microparticles express specific surface membrane protein with constituent phosphatidyl serine, a negatively charged phospholipid, and the particles are carriers carrying biologically active molecules and having proinflammatory, procoagulant and adhesion-promoting functions. However, few studies have focused on the role of MPs in ARDS. It has been shown that intravenous injection of particulate-induced endothelial cell-derived elevated BALF and plasma TNF-α and IL-1β levels, inducing acute lung injury in mice [2, 5]. In addition, in the bronchoalveolar lavage fluid (BALF) of patients with ARDS, the numbers of tissue factor-positive microparticles (TF-MP)
increased, suggesting that activation of alveolar epithelial cells may release microparticles or microparticles can be distributed to the lungs through the alveolar-capillary barrier [6]. Based on the current understanding of the pathophysiology of ARDS, ARDS is induced by the interaction among neutrophils, endothelial cells, alveolar epithelial cells, platelets and other cells as well as among TNF-α, IL-1β and other cytokines.

It is not known how the MPs of different origins differ in morphology and function based on their cell of origin. How microparticles play a role, depending on blood flow environment or vascular endothelial cells, remains unanswered. Therefore, the objective of the current study was to investigate the total plasma particles and the different phenotypes based on their cellular origin.

Methods

Animals and experimental groups

The study was approved by the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital. Eighteen healthy male Wistar rats of SPF grade, weighing approximately 200 g, were randomly divided into three groups (n = 6 in each) - no intervention group, saline control group, and ARDS group. For the saline control and ARDS groups, the animals were anesthetized using intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and fixed in a supine position. A neck incision was made along the midline of the neck to expose the trachea, followed by airway instillation of either 0.2 ml physiological saline or lipopolysaccharide (LPS) (10 mg/kg) (O55B5; Sigma-Aldrich, USA) dissolved in 0.2 ml saline for the saline control and ARDS group, respectively.

Collection of abdominal aortic blood and extraction of MP

Twenty four hours after intervention, animals were anesthetized and a midline abdominal incision was performed to expose abdominal aorta. Abdominal aortic blood were collected into 15 ml sterile centrifuge tube containing citrate anticoagulant and centrifuged at 1500 g for 20 minutes. 1 ml of plasma was drawn carefully by a micropipette into a 1.5 ml sterile Eppendorf tube and centrifuged at 13,000 g for 2 minutes to obtain the platelet-poor plasma (PFP) and the supernatant was transferred into a new sterile 1.5 ml Eppendorf tube and centrifuged at 21,000 g for 1 hour, and then the supernatant was discarded. Approximately 100 µl of the PFP left was washed and resuspended in 1 × PBS and stored at -80°C until further use.

MP detection through transmission electron microscopy

20 µl MP suspension from each group were separately placed on the copper mesh and allowed to stand for 5 minutes. Filter paper was used to absorb excess liquid followed by addition of 20 µl of phosphotungstic acid and a further incubation for 10 minutes before microscopy was performed.

MP detection through flow cytometry

30 µl of MP suspension from each group were stained with either (a) allophycocyanin (APC) annexin V, phycoerythrin (PE) CD61, and fluorescein isothiocyanate (FITC) CD45, (b) APC-annexin V and PE-CD54, and (c) APC-annexin V and the corresponding FITC and PE IgG isotype controls. All cells were resuspended in calcium-containing annexin V assay buffer and counterstained with 10 μg/ml propidium iodide (PI) (Roche Diagnostics, Indianapolis, IN) to allow exclusion of dead cells. At least 30,000 events were collected for each analysis. Data was analyzed using FlowJo version 9 (Tree Star, Ashland, OR). As the diameter of the MPs vary between 0.1-1 µm, in order to better determine its location, we used gating for 0.7-0.9 µm polystyrene microspheres. The establishment of the forward scattered light and side scattered light double logarithmic scatter plot (FSC-H × SSC-H) was done, and the detection threshold, gain and voltage of the flow cytometer were adjusted according to the positioning control, so that the 0.9 µm microspheres were clearly distributed in the FSC-H × SSC-H scatter plot (Figure 1A-C). Then a rectangular gate was built with 0.9 µm microspheres as upper limit to analyze membrane particles (Figure 1D). Annexin V+ indicated plasma microparticles from all sources, annexin V+CD61+ indicated platelet-derived microparticles, annexin V+CD45+ indicated leucocytes-derived microparticles, and annexin V+CD54+ indicated endothelial cells-derived microparticles (Figure 2).
Figure 1. Establishment of the forward scattered light and side scattered light double logarithmic scatter plot (FSC-H × SSC-H) was done according to the positioning control to adjust the detection threshold, gain and voltage of the flow cytometer, so that the microspheres clearly distributed according to size ~5 µm (A), 2.5 µm (B), and 0.7-0.9 µm (C). Measurement of the microspheres according to the 0.7-0.9 µm gating (D).
Results

Transmission electron microscopy (TEM) depicting the morphological characteristics of MPs

Since for nanometer-sized particles, ordinary electron microscope cannot observe its shape, we used TEM to observe the morphology of particles (Figure 3). In all three groups, 0.1-1 µm diameter spherical objects of unequal size (100-500 nm) were observed, and they had a typical structure of the lipid bilayer.

FACS analysis of MPs

There was no statistical significant difference among the experimental groups in the number of either total (MP) or platelet-derived (PMP) microparticles (Figure 4). However, in both leucocyte-derived (LMP) and endothelial-derived (EMP) microparticles, ARDS was significantly higher than the no intervention or saline control groups (P < 0.05) (Figure 4).

Discussion

Microparticles are small vesicles of diameter 0.1-1 µm, released from surfaces of activated or apoptotic cells. It has been confirmed in vivo and in vitro that microparticles may originate from endothelial cells, platelets, white blood cells, red blood cells, vascular smooth muscle cells, cardiac muscle cells, tumor cells, etc. [2-4, 7-10]. However, a clear definition of microparticles is still lacking; generally a microparticle is considered to harbor the following characteristics: (1) a spherical structure of diameter 0.1-1 µm; (2) associated with the cytoskeleton, the membrane has a phospholipid bilayer structure; (3) lack of nuclei or lack of the ability to synthesize proteins; (4) may be derived from different cells, and the expression of cell surface-origin specific antigens; and (5) high expression of phosphatidylserine [11].

During the isolation of MPs, it is imperative to remove the red blood cells, white blood cells, especially platelets in order to exclude interference of detection. Hence, we used centrifugation and filtration two ways. A lower centrifugal force (1,500 g for 20 minutes) was adopted to remove cells and debris and obtain platelet-rich red blood cells and white blood plasma. Some studies have adopted centrifugation at 13,000 g for 2 minutes to obtain platelet-poor plasma. Filtration to remove platelets also works and is especially useful where high speed centrifugation is not available; however, filtration is labor intensive and time consuming. Some studies have used 100,000 g for 60 minutes to centrifuge the platelet-poor plasma to obtain MPs. Even though such condition will yield maximal amounts of MPs, it might be contaminated with co-precipitation of exosomes (40-100 nm diameter). Hence, we opted for 21,000 g to avoid introduction of exosomes [12-17]. Furthermore, no statistically significant difference was observed in the number of extracted microparticles between using 20,000 and 100,000 g [18].

Microparticles in the blood can be detected by a variety of methods, mainly by flow cytometry, ELISA assay, solid phase capture measurement, scanning electron microscopy (SEM) and TEM microscopy. Wherein flow cytometric identification and enumeration of microparticles is standard for microparticles with diameter smaller than 1 µm, FACS also allows detection of the positive expression of phosphatidylserine (can be displayed by binding with fluorescent-labeled Annexin-V). Furthermore, antigen-antibody binding can also be used to determine the source of cells and identify different phenotypes of MPs, for example, CD235a and CD45 are detected to confirm erythrocyte-derived MPs and leukocyte-derived MPs, respectively [19, 20]. However, the method of flow cytometry is not perfect, due to the resolution limit of forward scatter, only MPs larger than 0.5 µm can be detected. ELISA method can also be used to analyze microparticles [21, 22] less than 0.5 µm by detecting phosphatidylserine or other specific antigens. The disadvantage though is that ELISA cannot measure the size, so that microparticles can not be separated...
Figure 3. Direct observation of microparticles with electron microscopy (A). Observation of microparticles by negative staining, showing microparticle diameter of 100-500 nm (B).
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From exosomes and apoptotic body area. In solid phase capture measurement, monoclonal antibody-annexin-V is used to bind phosphatidylserine on the phospholipid surface, microparticles are counted by measuring the expression of phosphatidylserine, but this method has the same disadvantages as ELISA.

By comparing the above several methods, it can be deduced that transmission electron microscope can be used to observe the morphological characteristics of microparticles. The microparticles observed have diameter of 0.1-1 μm and size heterogeneity, and have lipid bilayer structures, and the size and shape are similar to those reported in literature [5, 23]. In addition, we used the most widely used flow cytometry for detecting and counting, and selected different phenotype-specific markers to analyze MPs, i.e. CD45 for leukocyte-derived MPs [5, 20, 24-26], CD61 for platelet-derived MPs [26, 27], and CD54 for endothelial cell-derived MPs. We selected 0.7-0.9 μm microspheres as internal control gates to define the size of microparticles, thus effectively excluding the interference, and used standard microspheres for quantitative determination of the total number of microparticles. The above experiments strongly confirmed the presence of microparticles in the blood.

Recent studies have confirmed that many body fluids contain microparticles, such as blood, urine, pleural effusion, ascites, joint effusion, saliva, etc. [27-32], as well as platelets [33] where the number of microparticles is the most. Currently there are many reports of elevated microparticles inflammatory diseases, such as endothelial cell-derived microparticles in diabetes [34], psoriasis [35], multiple sclerosis [36], sepsis [37], systemic inflammatory response syndrome [38], and leucocyte-derived microparticles in anti-neutrophil cytoplasmic antibody-associated vasculitis (ANCA-associated vasculitis) [39], and diabetes [40]. We interestingly found significantly more LMPs and EMPs, but not PMPs in ARDS compared to healthy control; furthermore there was no difference in the total number of MPs between the experimental and control groups. This corroborates with a previous finding of more leucocyte- and neutrophil-derived MPs in bronchoalveolar lavage fluid in ARDS patients [41].

Our results indicate that leukocyte-derived and endothelial cell-derived microparticles may play an important role in ARDS. The expression of MPs under different pathological conditions is often different. The increases of endothelial cell-derived microparticles, leukocyte-derived microparticles and platelet-derived microparticles indicate endothelial injury, inflammation and coagulopathy, respectively. Thus, due to large individual differences in the level of microparticles, the monitoring of the change trends and the analysis of the phenotype of microparticles have more clinical significance, it will be important to study the inflammation response to further explore the role of these microparticles in ARDS.

Disclosure of conflict of interest

None.

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References


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