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Human microparticles generated during sepsis in patients with critical illness are neutrophil-derived and modulate the immune response

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Abstract

BACKGROUND—Microparticles (MPs) are 0.3 μ m to 1.0 μ m vesicles generated after cell activation or apoptosis that may play a role in the pathophysiology of sepsis. We sought to elucidate the role of MPs in patients with critical illness and hypothesized that MPs are generated at the site of inflammation and can modulate the immune response.

METHODS—Surgical patients with critical illness with ongoing sepsis were enrolled from the intensive care unit of an urban, Level I trauma center from March to June 2011. Abdominal washings and bronchoalveolar lavage fluid were collected from sites of inflammation. MPs were isolated using differential centrifugation, then characterized by flow cytometry. Immunologic assays were conducted by incubating neutrophil-derived MPs (NDMPs) with a human monocytic cell line (THP-1). A *p* value ≤ 0.05 was considered significant.

RESULTS—MPs were absent in noninflamed foci in patients, whereas NDMPs were present in locations of inflammation. NDMPs were added to cultured THP-1 cells to quantify immunomodulatory effects. THP-1 cells were able to phagocytose NDMPs. Cells that ingested NDMPs demonstrated increased activation. In contrast, bystander THP-1 cells without ingested NDMPs demonstrated decreased activation.

CONCLUSION—NDMPs are generated at the site of inflammation in patients with critical illness during sepsis. They have a divergent effect on the immune response by activating phagocytic cells and deactivating bystander cells. NDMPs may play an important role in regulating the inflammatory response to sepsis in patients with critical illness.

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AUTHORSHIP

P.C.P. participated in sample acquisition and processing and analysis of data, performed statistical analysis, and drafted the article. C.C.C. participated in study design and coordination, contributed to data interpretation, and helped draft the article. A.B.L. contributed to data interpretation, provided experimental supplies and equipment, and provided critical input into the article. T.A.P. participated in sample acquisition and data interpretation and provided critical input into the article. B.R.H.R. participated in study design, participated in sample acquisition, and provided critical input into the article. All authors read and approved the final article.

DISCLOSURE

The authors declare no conflict of interest.

LEVEL OF EVIDENCE—Prognostic/epidemiologic study, level III.**Keywords**

Microparticles; sepsis; immune system

BACKGROUND

Sepsis is the leading cause of morbidity and mortality in surgical intensive care units (ICUs).^{1,2} Despite recent advancements in treatment and management, 1.1 million hospitalized patients develop sepsis annually in the United States, with an accompanying annual mortality rate of 28.6%.² Sepsis and its associated complications remain an enormous economic burden on the health care system, with total annual costs exceeding US \$14.6 billion nationally.² Because of several cases, high mortality rates, and associated costs, sepsis continues to remain a serious concern to health care providers.³

At the onset, sepsis is characterized by a robust leukocyte recruitment and an overwhelming release of inflammatory mediators, which in turn are responsible for hypoperfusion and organ dysfunction. As sepsis progresses, a shift toward an antiinflammatory (immunosuppressive) state is observed clinically.⁴ An important hallmark of immune suppression is profound immune cell apoptosis. One consequence of this is the ingestion of apoptotic, phosphatidyl serine (PS)-expressing bodies by phagocytes and the subsequent secretion of anti-inflammatory cytokines such as transforming growth factor β , PGE₂, and interleukin 10.^{5,6} Thus, leukocyte apoptosis and phagocytosis can lead to a reduced number of cells available for mounting an antimicrobial response concomitantly with an increasing secretion of anti-inflammatory cytokines. This immune suppression, coupled with bacterial antibiotic resistance, represents a significant risk to increased mortality and morbidity for patients with critical illness or injury.

One possible hypothesized inflammatory mediator of infection is the microparticle (MP). MPs are derived from cell membranes and range in size from 0.3 μ m to 1.0 μ m. They have long been termed “platelet dust” and considered inert debris reflecting cellular activation or damage,^{7,8} but this is likely an overly simplistic characterization. More recently, they have been acknowledged as cellular effectors involved in cell to cell crosstalk and may represent novel markers of disease activity.⁷ The most abundant MPs in circulating blood are derived from platelets. In addition, MPs in the periphery can also arise from a variety of cell types such as lymphocytes, myeloid cells, and endothelial cells.⁷ MPs display membrane proteins implicated in a variety of fundamental processes and thus constitute a disseminated pool of bioactive effectors.⁸ They contain membrane, cytoplasmic, and nuclear constituents characteristic of their precursor cells and differ in size and composition from other subcellular structures such as apoptotic bodies and exosomes.⁷ They are thought to contribute to hemostatic and inflammatory responses, vascular remodeling and angiogenesis, cell survival, and apoptosis.⁹

MPs are possibly generated during sepsis because of leukocyte activation and apoptosis. We conducted a prospective, observational trial to characterize the type and amount of generated MPs in the inflamed foci of patients with critical illness or injury diagnosed with sepsis and

to determine whether these MPs participate in the development of immune suppression. We hypothesized that MPs are generated during sepsis and can further decrease leukocyte activation, contributing to immune suppression.

PATIENTS AND METHODS

Study Location and Patients

The study was conducted in the 34-bed surgical ICU of University Hospital in Cincinnati, Ohio, between March and November 2011. Three patients undergoing exploratory laparotomy for peritonitis, 4 patients undergoing elective abdominal surgery, and 33 surgical patients with critical illness or injury undergoing diagnostic fiberoptic bronchoscopy with bronchoalveolar lavage (BAL) based on an institutional protocol for the diagnosis of ventilator-associated pneumonia (VAP) were enrolled. All patients with peritonitis had a visceral perforation accompanied with a gross contamination of the abdomen and were considered septic ($n = 3$). Patients undergoing elective abdominal surgery were used as a nonseptic control ($n = 4$). Patients were diagnosed with sepsis by the treating surgical intensivist following the American College of Chest Physicians/Society of Critical Care Medicine definition, defined as those patients meeting two of the four systemic inflammatory response syndrome criteria with an infectious process.¹⁰ Indications for BAL for suspected VAP were defined by ICU guidelines as the appearance of a new or changing infiltrate on chest radiograph or macroscopically purulent sputum plus at least two of the following: temperature higher than 38°C or lower than 36°C, white blood cell count higher than 12,000/mm³ or lower than 4000/mm³, 10% bands, heart rate higher than 90 beats per minute, respiratory rate higher than 20 breaths per minute, or Paco₂ lower than 32 mm Hg.^{11–13} Two BAL samples from Jung donors were obtained and used as an uninfected control in the subset of patients with suspected pneumonia.

Study Design and Aims

This was a pilot study to evaluate the role of MPs in the inflamed foci of patients with sepsis or infection. The specific aims of the study were (1) to determine the presence of MPs in inflamed foci, (2) to characterize and quantify these MPs, and (3) to determine if MPs can alter the inflammatory process.

Fluid Collection

Peritoneal waste fluid was collected in sterile 50-mL conical tubes from patients that underwent exploratory Japa rotomy or elective abdominal surgery in which abdominal irrigation was performed. Fluid was immediately placed on ice for MP analysis within 2 hours of collection time.

All study patients with a clinical suspicion of VAP underwent diagnostic fiberoptic bronchoscopy with BAL. All BAL procedures were performed in a uniform manner by the treating surgical intensivist as part of ICU clinical practice guidelines. The bronchoscope was advanced into the lung segment wherein radiographic changes were seen. After the bronchoscope was wedged into the appropriate lung segment, 20 mL of sterile saline was

instilled into the lung as a wash. The waste BAL effluent was collected in a sterile specimen trap and immediately placed on ice for MP analysis within 2 hours of collection time.

Abdominal fluid and BAL fluid (BALf) samples were collected as unidentifiable surgical waste that could not be linked back to the investigator and was thus exempt from the institutional review board oversight as outlined in the NIH human research institutional review board exemption policy 46.101.

Cell and MP Isolation

Either abdominal fluid or BALf (between 2 mL and 20 mL) was obtained from patients meeting the study previously mentioned criteria. All fluid was kept on ice until time of processing. The fluid samples were initially filtered using a 150- μ m, sterilized stainless steel mesh into a sterile 50-mL conical tube. Cells in the fluid were pelleted after a 10-minute 450g centrifugation. The supernatant was then centrifuged for 10 minutes at 9900g to remove platelets. The platelet-free supernatant was centrifuged for 20 minutes at 16,000g to pellet MPs. The MP pellet was resuspended in 500 μ L of sterile phosphate-buffered saline (PBS) and centrifuged for 20 minutes at 16,000g. The MP pellet was then resuspended in 100 μ L of sterile PBS for analysis.

Flow Cytometry for Surface Staining

The analysis of cell surface antigen expression as previously described^{14,15} was performed on abdominal fluid and BALf. Flow cytometry data acquisition and analysis were performed on an LSR II using FACS Diva software (BD Biosciences, Mountain View, CA). The antibodies used were as follows: CD66b (clone G 10F5; BD Biosciences), CD105 (clone 43A3; BioLegend), CD41a (clone fFIP8; BD Biosciences), CD235 (clone HIR2; BioLegend), CD14 (clone M5E2; BioLegend), CD11b (clone MI/70; BioLegend), HLA-Dr (clone L243; BD Biosciences), CD80 (clone L307.4; BD Biosciences), and CD86 (clone 2331; BD Biosciences).

THP-1 Phagocytosis and Activation Determination

Neutrophil-derived MPs (NDMPs) isolated from infected foci were labeled with 5-(and-6)-carboxyfluorescein diacetate (CFSE) (Invitrogen, Carlsbad, CA). An equal volume of 10 mM CFSE was diluted to 1:5000 in PBS just before use. MPs and CFSE were mixed in a 1:1 volume ratio and incubated for 8 minutes at room temperature on a rocker. An equal volume of FBS was added and allowed to sit for 1 minute at room temperature. Next, an equal amount of sterile K5 media was added to the mixture and centrifuged at 16,000g for 20 minutes to pellet MPs. CFSE-labeled NDMPs were incubated with THP-1 cells (ATCC, Manassas, VA), a human monocytic cell line, at 37°C in a CO₂ incubator overnight. THP-1 cells were collected and analyzed for macrophage activation as determined by HLA-Dr, CD80, and CD86 mean fluorescence intensity (MFI).

To determine phagocytic ability, ND MPs were labeled with CFSE as previously described and incubated with THP-1 cells. After 18 hours, cells were collected and mixed with opsonized Fluoresbrite Polychromatic Red Plain Microspheres 1.0 μ M (Polysciences, Warrington, PA). The suspension was incubated at 37°C for 30 minutes. After the

incubation period, the phagocytosis was stopped by washing twice. Samples were run on a Becton Dickinson LSR II to determine MFI of(+)CFSE THP-1 and (-)CFSE THP-1 cells as a measure of phagocytosis.

Statistical Analysis

Statistical comparisons were performed using Student's *t* test (two groups) or one-way ANOVA with Holm-Sidak post hoc test (more than two groups) using StatView 3.5 (SAS Institute, Cary, NC). The mean and standard error of the mean were calculated in experiments containing multiple data points; $p < 0.05$ was considered statistically significant.

RESULTS

MPs Are Observed at the Infectious Foci in Humans and Are Neutrophil Derived

We initially hypothesized that MPs were generated in humans during sepsis. We obtained abdominal fluid from patients with sepsis with peritonitis as well as BAL from those with suspected pneumonia as representative of inflamed foci from the abdomen and lungs, respectively. Abdominal fluid from patients undergoing elective surgery and BAL from lung donors were used as nonseptic controls. These samples underwent differential centrifugation to selectively isolate MPs. We further labeled the MPs with antibodies against CD66b (neutrophil marker), CD41 (platelet marker), CD105 (endothelial cell marker), and CD235 (red blood cell marker). As shown in Figure 1, we used latex beads of known size for flow cytometric analysis for size gating between 0.3 μm and 1.1 μm to identify MPs. Further, we determined that an increased number of MPs are present in the abdomen of patients with peritonitis when compared with healthy controls and that these MPs were derived primarily from neutrophils (Fig. 2). In addition, there was no distinct, identifiable MP population present in the abdominal fluid of patients without sepsis (Fig. 2). We did not observe MPs derived from any other cell type (data not shown). Thus, NDMPs are the predominant MP generated at the infectious focus during sepsis.

NDMPs Are Ingested by Monocytes and Have a Divergent Effect on the Immune System

Previous reports have indicated that MPs express the apoptosis marker, PS, on the extracellular leaflet of the plasma membrane.^{16,17} PS is also a signal for phagocytosis (as reviewed by Ayub and Hallett¹⁸). This led us to examine whether NDMPs are actively phagocytosed. We incubated fluorescent, CFSE-labeled NDMPs with the human monocytic cell line, THP-1. We gated on CD14, CD11b-positive THP-1 cells to identify monocytes and observed an increase in the mean fluorescence of the FL-1 (CFSE-specific) channel from 7.17% to 61.48%, suggesting NDMP ingestion by THP-1 cells (Fig. 3).

As demonstrated in Figure 3, THP-1 cells phagocytose NDMPs. To further determine the effect of this process on cell function, we incubated THP-1 cells with CFSE-labeled NDMPs for 24 hours. The use of CFSE-labeled NDMPs allowed us to discriminate between THP-1 cells that had ingested NDMPs versus those that did not (bystander THP-1 cells). To determine the macrophage activation status, the cell surface markers HLA-Dr, CD80, and CD86 were fluorescently quantified for THP-1 cells that ingested MPs, bystander cells, and

untreated THP-1 cells. We observed cells that had ingested ND MPs had increased activation as seen by an increase in the MFI of HLA-Dr, CD80, and CD86 as compared with both untreated and bystander THP-1 cells (Fig. 4). Further, we observed that bystander cells demonstrated decreased activation as seen by a decrease in these surface markers (Fig. 4). Given these data, NDMPs appear to have a divergent effect on macrophage phenotype.

We next sought to evaluate the functional effect of the ingestion of NDMPs on THP-1 cells. THP-1 cells were incubated with CFSE-labeled NDMPs. After 18 hours, the cultured cells were incubated with red fluorescent microspheres as described in the Patients and Methods section, and phagocytic ability was evaluated with flow cytometry. Cells that had ingested ND MPs showed an increased phagocytic ability when compared with bystander cells (Fig. 5).

DISCUSSION

In the present study, we tested the hypothesis that MPs are generated at the site of inflammation during sepsis and affect the immune system. First, we demonstrated that MPs are generated in the inflamed abdomen or lungs of patients with sepsis and that these MPs are mainly of neutrophil derivation. Previous studies have shown that MPs contain PS on their cell surface, a signal for phagocytosis.^{16–18} Our data demonstrate that NDMPs are indeed phagocytosed by THP-1 cells and that this phagocytosis has a divergent, and potentially important, effect on the immune system. Cells that had ingested NDMPs had increased activation and phagocytic ability as compared with both cells not exposed to NDMPs and bystander cells. Further, we observed that those cells that did not ingest ND MPs had decreased activation and phagocytic ability. Taken together, these data suggest that ND MPs are present at the site of inflammation in patients with critical illness and affect the immune response to infection.

The role of NDMPs in sepsis is poorly understood. Recently, it was observed that activated human neutrophils released NDMPs in an inflammatory environment.¹⁹ Subsequently, it was published that NDMPs were shed during neutrophil apoptosis.²⁰ Increased NDMPs have been observed in patients with vasculitis, those undergoing hemodialysis, and those with cystic fibrosis.^{21,22} Altogether, the generation and effect of ND MPs during sepsis is mainly of an observational nature and is poorly understood. In the current study, we have demonstrated that NDMPs not only are generated at the site of infection and inflammation in patients with sepsis but also modulate the immune response to sepsis. This provides important insight into the possible role of ND MPs in the disordered immune response during sepsis.

Although the mechanism of NDMP generation is unknown, cell activation and apoptosis have been proposed as possible processes (as reviewed by Montoro-Garcia,²³ Shcherbina and Remold-O'Donnell,²⁴ and Dale and Friese²⁵). During cell activation, remodeling of the plasma membrane can take place. This membrane modification can cause bleb formation, leading to the extrusion of MPs that contain surface proteins and other contents of the originating cell.⁷ MP release is also associated with apoptosis.⁷ MP formation occurs late in the cell death process and may occur at the same time as cell fragmentation and the

formation of apoptotic bodies. These differences in the mechanisms of MP formation between cell activation and death likely account for the variations in MP size as well as macromolecular surface and internal compositions.⁷

The phagocytosis of ND MPs by monocytes may play a role in the shift toward an anti-inflammatory state observed in the later stages of sepsis. Early on in sepsis, the activation of neutrophils is the likely mechanism of NDMP generation. As sepsis persists and immune suppression dominates, cells undergo apoptosis, promoting further generation of NDMPs. These NDMPs are phagocytosed by monocytes, causing the amplified activation of the ingesting leukocytes and the deactivation of surrounding cells. The activation of these cells likely exacerbates immune suppression by secreting antiinflammatory cytokines such as transforming growth factor β , PGE2, and interleukin 10. Thus, leukocyte apoptosis and ingestion of NDMPs can lead to fewer cells available for mounting an antimicrobial response as well as to increasing levels of anti-inflammatory cytokines. The exacerbation of immune suppression coupled with the growing presence of pan-resistant microbes in the ICU setting represents a significant risk to the morbidity and mortality of patients with critical illness or injury.

Limitations to this study include a small sample size of patients with abdominal sepsis. A larger patient group would allow us to determine variation in NDMP generation with regard to variables such as severity of sepsis and type of injury. In addition, all samples were collected as surgical waste. A larger, prospective trial will help characterize MPs in subsets of patients with sepsis as well as correlate MP type and numbers with comorbidities. Finally, this study is also limited by its in vitro nature. The effect of NDMPs on monocytes in culture may be insignificant or not present in vivo. However, such observations suggest that MPs are a possible inflammatory mediator of infection and warrant further investigation.

In conclusion, we report significant findings that advance the field of study on MPs in sepsis. We demonstrated that ND MPs are generated in inflamed foci of patients with sepsis and have a divergent effect on the immune system. Further studies investigating the mechanisms underlying NDMP formation and the effect of their formation in vivo and in vitro are needed. Sepsis is a dynamic process that is complicated by an interplay of pro-inflammatory and anti-inflammatory mediators. A delicate balance of these two opposing forces allow for the immune system to mount an effective response to infection and inflammation. When this is disrupted, infection goes uncontrolled and patients incur morbidity and mortality. NDMPs appear to play a role in this balance, offering itself as a possible target for future therapeutic intervention as well as a potential clinical predictor of patient morbidity and outcome. Looking forward, these findings suggest that NDMPs are a novel bioactive mediator of sepsis that once studied in further detail may help thwart the ongoing burden sepsis has on our health care system.

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Figure 1.

Sizing and gating strategy for the identification of MPs. (A) Latex beads of a predetermined size were used to set the forward and side scatter voltages to best distinguish particles ranging from 0.3 μm to 3.0 μm . (B) Representative gating demonstrating sizing strategy to detect MPs.

Figure 2.

NDMPs are generated in the infected foci of patients with critical illness and are the predominate type of MP identified. (A) Abdominal irrigation fluid was obtained from patients either undergoing exploratory laparotomy for peritonitis or elective surgery. BAU was obtained from patients with suspected pneumonia or lung donors without pneumonia. MPs were characterized as described in the Patients and Methods section. (B) Flow cytometric analysis demonstrates significantly increased percent NDMPs in abdominal infections. Analysis on BAU was not included in this data set. $n = 3$ for infected abdomens, and $n = 4$ for uninfected abdomens.

Figure 3.

NDMPs are binding to or phagocytosed by THP-1 cells. Human NDMPs were fluorescently labeled with CFSE and then incubated for 18 hours with THP-1 cells, a human monocytic cell line. THP-1 cells were gated on CD14 and CD11b surface expression levels, and CFSE fluorescence was determined by flow cytometry as described in the Patients and Methods section. Five independent samples were assayed. Data are expressed as the mean (SEM). * $p < 0.05$ as determined by Student's t test.

Figure 4.

THP-1 cells incubated with NDMP demonstrate divergent activation. Fluorescently labeled NDMPs from patients with critical illness were incubated 18 hours with THP-1 cells. Untreated, (+)CFSE THP-1, and (-)CFSE THP-1 cells were analyzed by flow cytometry for (A) HLA-Dr, (B) CD80, and (C) CD86 surface expression levels. Six independent samples were assayed, * $p < 0.05$ as compared with untreated and bystander, and # $p < 0.05$ as compared with ingested as determined by ANOVA pairwise comparison.

Figure 5.

Ingestion of NDMPs increases the phagocytic ability of THP-1 cells. THP-1 cells were incubated with CFSE-labeled NDMPs. After 18 hours, the phagocytic ability of (+)CFSE THP-1 and (-)CFSE THP-1 cells was determined as describe in the Patients and Methods section. Data are expressed as the mean (SEM). * $p < 0.05$ as determined by Student's t test.