Optimising diagnosis and treatment of coagulopathy in severely injured trauma patients
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ENDOGENOUS MICROPARTICLES DRIVE THE PROINFLAMMATORY HOST IMMUNE RESPONSE IN SEVERELY INJURED TRAUMA PATIENTS

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ABSTRACT

Introduction: Severe trauma affects the immune system, which in its turn is associated with poor outcome. The mediators driving the immune responses in trauma are largely unknown. The aim of this study was to investigate the role of endogenous microparticles (MPs) in mediating the immune response following severe trauma.

Methods: A prospective, observational substudy of the ACIT II (Activation of Coagulation and Inflammation in Trauma II) study was performed at our academic level I trauma centre. Adult multiple-trauma patients with an injury severity score of 15 or higher were included between May 2012 and June 2013. Ex vivo whole-blood stimulation with lipopolysaccharide was performed on aseptically collected patient plasma containing MPs and in plasma depleted of MPs. Flow cytometry and transmission electronic microscopy were performed on plasma samples to investigate the numbers and cellular origin of MPs. Healthy individuals served as a control group.

Results: Ten trauma patients and 10 control subjects were included. Trauma patients were significantly injured with a median injury severity score of 19 (range, 17-45). Patients were neither in shock nor bleeding. On admission to the hospital, the host response to bacterial stimulation was blunted in trauma patients compared with control subjects, as reflected by decreased production of interleukin 6 (IL-6), IL-10, and tumour necrosis factor α (P< 0.001). In trauma patients, MP-positive plasma was associated with a significantly higher synthesis of IL-6 and tumour necrosis factor α compared with plasma depleted from MPs (P = 0.047 and 0.002, respectively). Compared with control subjects, the number of circulating MPs was significantly decreased in trauma patients (P = 0.009). Most MPs originated from platelets. Multiple cellular protrusions, which result in MP formation, were observed in plasma from trauma patients, but not in control subjects.

Conclusions: On admission, trauma patients have a reduced immune response toward endotoxin challenge, which is, at least in part, mediated by MPs, which circulate in low numbers and in early stages. Most MPs originate from platelets, which indicates that these cells may be the most important source of MPs involved in initiating an inflammatory host response after injury.
INTRODUCTION

Traumatic injuries are responsible for a high morbidity and mortality worldwide. Multiple trauma affects the immune system in a complex way. Currently, it is thought that both hyperinflammation and immunosuppression can occur following severe trauma. On the one hand, hyperinflammation is associated with a cytokine storm causing a systematic inflammatory response syndrome (SIRS), which is strongly associated with the occurrence of multiple organ failure and mortality. On the other hand, immunosuppression is an early event following trauma, characterized by a decreased ability of immune cells to respond to bacterial antigens and is associated with an enhanced risk of developing hospital-acquired infections and mortality. Therefore, understanding the immune response is important to improve outcome of trauma patients. However, mediators of host responses after injury are largely unknown.

Microparticles (MPs) are vesicles shed into the bloodstream by cells under conditions of stress. Microparticles can originate from various cells and are thought to play an important role in intercellular communication by activating circulating monocytes and leukocytes with subsequent production of inflammatory cytokines. In line with this, previous studies have reported increased numbers of circulating MPs in patients with SIRS and sepsis. Also, increased numbers of MPs, mainly originating from platelets, are observed in trauma patients with SIRS. It is unknown whether increased numbers of circulating MPs are cause or effect of the host immune response after injury. If MPs mediate the immune response after injury, they could be useful biomarkers or therapeutic targets to influence the immune system and improve the long-term outcome of trauma patients.

The aim of this study was to investigate the role of MPs in mediating the immune response following trauma. We determined the numbers and cellular origin of MPs following severe trauma and investigated the role of MPs in the generation of a host immune response to a bacterial antigen.

MATERIALS AND METHODS

This study was a substudy of the ongoing Activation of Coagulation and Inflammation in Trauma II study (ACIT II, UKCRN ID: 5637), which is a prospective observational multicentre study on mechanisms of coagulopathy and inflammation in trauma patients. This substudy was performed at our academic level I trauma centre after approval by the local ethics committee.
Ten adult severely injured multiple-trauma patients with an injury severity score (ISS) of more than 15 who were admitted to the emergency department (ED) at the Academic Medical Center between May 2012 and June 2013 were studied. Patients who were transferred from other hospitals, who presented more than 2 h after injury on the ED, who were using anticoagulant medication, or for whom no informed consent was obtained were excluded. Patient demographics, mechanism of injury, prehospital fluids, Glasgow Coma Scale, injury severity score (ISS), systolic blood pressure, heart rate, haemoglobin, prothrombin time, platelet count, and leukocytes were recorded on the ED. Furthermore, data about the length of stay and in-hospital mortality were collected. Healthy volunteers of both sexes (aged 20-35 years) served as a control group. Written informed consent was obtained from each patient and control subject. When the patient was unconscious, written informed consent was obtained from a legal representative.

**Blood samples**
On admission to the ED, blood samples were collected into buffered trisodium citrate vacutainer tubes (2.7 mL; Becton Dickinson, Plymouth, UK). Within 15 min after blood sampling, the collection tubes were centrifuged to prepare platelet-free plasma (1,750 RCF, 18°C, 10 min). The upper two-thirds of this plasma was collected and centrifuged again. The obtained platelet-free plasma was stored as 250 2L aliquots at -80°C until time of assay. Blood sampling procedures were identical for control subjects.

**Cytokine production in response to lipopolysaccharide stimulation**
Prior to centrifugation, samples were thawed on melting ice for 1 h. Microparticles were pelleted by ultracentrifugation (50,000 revolutions/min, 20°C for 1h). After centrifugation, the MP-depleted (MP-) supernatant was removed and used as a control. The MP-containing pellet was used as MP-positive (MP+) samples.

To perform whole-blood stimulation, heparin-anticoagulated blood was collected from a healthy donor. To study the effect of MPs, 250 µL whole blood was 1:1 diluted with RPMI medium supplemented with glutamine 0.3 g/L to which either 50 µL trauma plasma containing MPs (MP+) or plasma depleted from MPs (MP-) pellet was added as well as 5 µL lipopolysaccharide (LPS) (1 ng/mL; Sigma-Aldrich, Steinheim, Germany) and incubated at 5% CO2 at 37°C for 24 h. In addition, whole blood was stimulated with LPS (1 ng/mL) only as a positive control (LPS) and with RPMI medium as a negative control (RPMI). In all stimulation assays, similar volumes were maintained. Whole-blood stimulation procedures were identical for control subjects.

After 24 h of incubation, blood samples were centrifuged (600 revolutions/min, 18°C, 10 min). The upper two-thirds of the plasma was collected and stored at -80°C. Interleukin 6 (IL-6), IL-10, and tumour necrosis factor-α (TNF-α) were measured in the
plasma samples by enzyme-linked immuno-sorbent assay, according to instructions of the manufacturer (R&D Systems, Abingdon, United Kingdom).

To examine the cytokine synthesis in plasma from trauma patients compared with control subjects, results from the whole-blood stimulation of MP+ and MP- samples were combined. Subsequently, to examine the effect of MPs on the cytokine synthesis in trauma patients or control subjects, MP+ samples were compared with MP- samples.

**Number and origin of MPs**

Flow cytometry with cell-specific markers was used to determine the origin of MPs. Fluorescein isothiocyanate (FITC)-labelled CD144 (Enzo Life Science, Farmingdale, NY) was used to determine endothelial-derived MP, FITC-labelled anti-CD235a (Dako, Glostrup, Denmark) for MP derived from erythrocytes, phycoerythrin-labeled CD61 (Becton Dickinson, San Jose, Calif) for MP derived from platelets, and FITC-labelled CD62p (Beckman Coulter, Brea, Calif) for MP derived from activated platelets. As negative controls, immunoglobulin G1 (IgG1)-phycoerythrin (Becton Dickinson; control for CD61), IgG1-FITC (Becton Dickinson; control for CD235a and CD62p), and IgG-FITC (Immuno Quality Products, Groningen, The Netherlands; control for CD144-FITC) were used. Thawed plasma from patients and control subjects (5 µL) was added to phosphate-buffered saline/citrate buffer (35 µL) supplied with antibody (5 µL) against a cell-specific antigen. The mixture was incubated for 15 min at room temperature, in the dark. Then, phosphate-buffered saline/citrate buffer (900 µL) was added, and samples were analysed on a FACS (Fluorescence-Assisted Cell Sorting) Calibur (Cellquest version 4.02; Becton Dickinson) for 1 min. The number of MPs per millilitre was calculated with the formula: \( N \times \left( \frac{\text{total volume (950 µL)}}{\text{volume used by FACS (60 µL)}} \right) \times \left( \frac{1,000}{5} \right) \).

**Morphology of MPs**

The morphology of MPs was examined by transmission electron microscopy. Size exclusion chromatography was used to isolate MPs from plasma as described before, and fractions 9 and 10 were used. Then, MPs were stained with anti-CD235a, anti-CD62p, anti-CD61, anti-CD142, anti-CD62p, or anti-CD42b as described before. In addition, MPs were stained with gold-labelled annexin V (gift from Alain Brisson, University of Bordeaux) as described before, but without the use of PPACK.

**Statistical analysis**

Normality for continuous variables was tested by visual inspection of histograms and by Kolmogorov-Smirnov test. Results are expressed as either mean ± SD or as median ± interquartile range (IQR), depending on the distribution of the variables. A Student t test was performed when normality assumption was obtained; otherwise, a Mann-Whitney U test was used to test for difference in cytokine synthesis between trauma
patients and control subjects. To test for difference between MP+ samples and MP-
samples, Wilcoxon signed rank test was used when normality assumption was not
obtained; otherwise, a Kruskal-Wallis test was performed. Spearman > was used to
examine the correlation between the number of MPs and the synthesis of ILs. P< 0.05
was considered to be statistically significant. Statistical analyses were done in IBM SPSS
Statistics 21 (IBM Corp., Chicago, Ill).

RESULTS

Patients enrolled in this study were severely injured as demonstrated by a high ISS.
No bleeding patients or patients with a haemorrhagic shock were included (Table 1).
All patients sustained a blunt trauma. Two patients died in the hospital because of
traumatic brain injury. The median length of hospital stay was 9 days. Hospital-
acquired infections were not observed, and transfusion of blood products was not
required.

| TABLE 1: Characteristics of trauma patients (N=10) |
|-----------------|-----------------|
| Age, median (IQR) | 60 (43-69) |
| Gender | Male, n (%) |
|       | 6 (60) |
| Mechanism of injury, n (%) | Fall from height |
|       | 5 (50) |
|       | Traffic |
|       | 5 (50) |
| ISS, median (IQR) | 19 (17-45) |
| AIS, n (%) | Head |
|            | 5 (50) |
|            | Face |
|            | 5 (50) |
|            | Chest |
|            | 4 (40) |
|            | Abdomen/Pelvis |
|            | 2 (20) |
|            | Extremities |
|            | 6 (60) |
|            | External |
|            | 2 (20) |
| Pre-hospital fluid administration ml, median (IQR) | 0 (0-500) |
| Systolic blood pressure mmHg, median (IQR) | 137 (115-155) |
| Heart Rate bpm, median (IQR) | 83 (63-112) |
| Hemoglobin, g/dl, median (IQR) | 13.3 (11.8-14.6) |
| Leukocytes x 10^9/L, median (IQR) | 9.0 (7.4-11.7) |
| Platelet count x 10^9/L, median (IQR) | 203 (167-264) |
| PT sec, median (IQR) | 11.5 (11.1-12.6) |
| Length of stay days, median (IQR) | 9 (2-23) |
| In-hospital mortality , n (%) | 2 (20) |

Patient characteristics and first laboratory results on the emergency department are shown.
ISS= injury severity score, AIS= abbreviated injury score, PT=prothrombin time.
Cytokine production in response to LPS stimulation

Trauma significantly abrogated the host response to LPS, as demonstrated by reduced levels of all measured cytokines in plasma from trauma patients compared with plasma from control subjects and compared with LPS control (Fig. 1). These results suggest a decreased immune response in trauma patients.

FIGURE 1: Cytokine synthesis in trauma patients and control subjects after whole-blood stimulation with LPS. Lipopolysaccharide and RPMI served as a positive and negative control, respectively.

In this study, we included five multiple-trauma patients with traumatic brain injury and five multiple-trauma patients without traumatic brain injury. Stimulation with plasma from patients with brain injury produced significantly lower levels of IL-10 (384 ± 159 vs. 537 ± 66 pg/mL, P = 0.005) and TNF-α (201 ± 120 vs. 338 ± 70 pg/mL, P = 0.008) compared with plasma from patients without brain injury, whereas IL-6 production did not differ (11,978 ± 5,201 vs. 9,038 ± 2,799 pg/mL, P = 0.151).

To determine the role of MPs in the host response, whole blood was incubated with LPS and plasma either containing MPs or depleted from MPs from trauma patients and control subjects. In trauma patients, IL-6 and TNF-α production in response to LPS was decreased in plasma depleted of MPs compared with plasma containing MPs. In control subjects, depletion of MPs resulted in a decreased IL-6 production. No significant difference was observed in IL-10 and TNF-α synthesis (Fig. 2).

FIGURE 2: Cytokine levels of MP+ plasma versus MP- plasma after whole-blood stimulation with LPS in control subjects and trauma patients. Micro-particles have a proinflammatory effect in trauma patients; proinflammatory cytokines IL-6 and TNF-α are both significantly increased.
Number and origin of MPs

The median concentration of MPs was reduced by approximately 50% in trauma patients (4.2 (2.3-6.7) x 10^6) compared with healthy volunteers (10.3 [7.0-14.2] x 10^6, P = 0.009). Microparticles in trauma patients originated particularly from platelets and less frequently from erythrocytes and endothelial cells, which was comparable to control subjects. Also in these cell subpopulations, the number of platelet and activated platelet MPs was significantly higher in control subjects compared with trauma patients (Fig. 3). No difference in the number of erythrocyte MPs and endothelial MPs was observed (P = 0.529 and 0.631, respectively). Of note, the numbers of MPs originating from endothelial cells was negligible.

FIGURE 3: Origin of MPs in trauma patients and control subjects. The numbers of platelet and activated platelet-derived MPs were significantly lower in trauma patients compared with control subjects (both P < 0.001).

In addition, a significant correlation was observed between the total number of MPs and the amount of IL-6 and TNF-α synthesis (P = 0.007 and 0.005). In particular, the number of platelet- and activated platelet-derived MPs correlated with IL-6 (P< 0.001 and 0.018) and TNF-α production (P = 0.005 and P = 0.017). No correlation was found between the synthesis of ILs and the number of erythrocyte and endothelial- or activated endothelial cell-derived MPs.

Morphology MPs

Transmission electron microscopy images of plasma from two trauma patients visualized not only MPs (cup-shaped, white arrow) but also other structures with a narrow protrusion (Fig. 4), which were described before as tethers and FLIPRs (flow-induced protrusions)\textsuperscript{27, 28}. The FLIPR structure stained positive with an anti-CD235a antibody, but not with antibodies against CD62p, CD61, CD142, CD62p, or CD42b, indicating that these structures originated from erythrocytes.
FIGURE 4: A and B, Besides MPs (cup-shaped, white arrow), cells with protrusions were observed in fresh plasma samples from trauma patients. Fragmentation of the protrusions will result in the development of MPs. C, Antibody CD235a expressed by erythrocytes bound to the cells with protrusions (black stains), indicating the erythrocyte origin of these cells. D, The cells with protrusions stained negative with IgG1-FITC (negative control).

DISCUSSION

On admission, trauma patients have a reduced immune response toward endotoxin challenge compared with control subjects, which is associated with a reduced number of circulating MPs, suggesting that these MPs drive the synthesis of proinflammatory cytokines in response to LPS. Most circulating endogenous MPs are derived from platelets, which indicates that these cells may be the most important source of MPs involved in host response after injury.

Cytokine production in response to LPS stimulation
Stimulation of plasma from trauma patients resulted in decreased cytokine levels compared with stimulation with plasma from control subjects. Decreased cytokine production was not caused by plasma per se, as addition of plasma from control subjects
to the assay rather seems to enhance cellular response. A reduced host response in trauma has been found before\textsuperscript{13}. It is hypothesized that a reduced cellular immune response in trauma patients toward endotoxin challenge is caused by exhaustion of immune cells, termed immunoparalysis. This may hold in particular for traumatic brain injury patients. It is known that in these patients the systemic innate immune response is diminished\textsuperscript{14, 29, 30}. This is confirmed by our results, which show that the host response is even more suppressed in patients with traumatic brain injury.

Mediators of the host response are unknown. We show here that in trauma, the immune response to LPS is, at least in part, mediated by endogenous MPs. This is in line with previous studies, in which it has been proposed that MPs derived from leukocytes circulate in low levels in control subjects and become up-regulated after systematic inflammation or in sepsis. Microparticles activate circulating cells and stimulate production of proinflammatory cytokines such as IL-1\(\beta\), IL-6, TNF-\(\alpha\), and IL-8\textsuperscript{7, 19-21, 24, 25, 28, 31}. Of interest, our data suggest that the stimulating effect of MPs on immune cells is a physiological response, given that MPs derived from trauma patients as well as from control subjects stimulate cytokine production. Whether a persistent depletion of MPs contributes to an increased risk of nosocomial infection following trauma remains to be determined.

**Number and origin of MPs**

The number of circulating MPs in trauma was significantly decreased compared with control subjects. This is in contrast to previous studies, in which a higher number of circulating MPs was observed in trauma patients with SIRS compared with control subjects\textsuperscript{23, 24}. A possible explanation for the decreased number of MPs in trauma patients may be activation of cells by MPs, resulting in the formation of complexes be-tween MPs and other cells. Complexes will not be diagnosed as MPs by flow cytometry, which may be a limitation of our study. Furthermore, the timing of blood draw in previous studies varied between 1 and 5 hospital days. As the turnover of MPs is highly dynamic, particularly following trauma, this may account for contrasting results between studies\textsuperscript{23-25}.

Most MPs in the severely injured trauma patients included in this study were derived from platelets. This is similar to previous studies in trauma patients with SIRS or coagulopathy in which MPs also originated from platelets\textsuperscript{23, 32}. Together, this suggests that platelet MPs may be the most important source of MPs involved in initiating an inflammatory host response after injury. Recently, in an ex vivo study, it was shown that activated platelets form protrusions (FLIPRs), which fragment resulting in platelet-derived MPs. These MPs could subsequently activate monocytes and neutrophils by
upregulation of adhesion molecules on the surface of endothelial cells, mediating a proinflammatory response\textsuperscript{28,33}. In line with this, the number of platelet-derived MPs correlated with the host response in this study. Follow-up studies are required to investigate the immunoactivity of platelets following trauma in more detail.

Given that patients sustained severe trauma with tissue injury, we expected to also find endothelial-derived MPs. However, the numbers of endothelial-derived MPs were low. An explanation may be that patients in this study were not bleeding, and hence endothelium was not disrupted. It is possible that bleeding patients display another profile of endogenous MPs.

**Morphology MPs**

When we investigated MP morphology in fresh plasma samples from trauma patients, we detected large numbers of cells with protrusions. These cells were not present in control subjects. The cells with protrusions in this study were derived from erythrocytes and could fragment into and thus are precursors of erythrocyte-derived MPs. We did not find platelet-derived protrusions at this time point. The consequence of finding large numbers of cells with protrusions cannot be dissected from our study, which is a limitation. We hypothesize that the development of an erythrocyte-derived MP storm may occur in time, with subsequent activation of leukocytes and monocytes with ensuing progression toward a proinflammatory response. However, this remains to be determined, as we did not measure host response at different time points.

Another limitation of this study is that flow cytometry detects only a minor fraction of all circulating extracellular vesicles such as MPs\textsuperscript{34}. However, currently, flow cytometry is the standard method to detect single MPs. More sensitive technology may improve detection limits. Furthermore, we have investigated the role of MPs in mediating the immune response following trauma in a selected group of blunt trauma patients as patients had significant injury, but patients were neither in shock nor bleeding.

In conclusion, severely injured trauma patients have a reduced immune response toward endotoxin challenge on ad-mission, which is strongly associated with decreased levels of circulating MPs. In these patients, MPs are able to drive the synthesis of proinflammatory cytokines and could abrogate the immunoparalysis. Most MPs are derived from platelets, which indicates that these cells may be the most important source of MPs involved in the host response after injury.
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