Microparticle generation and leucocyte death in Shiga toxin-mediated HUS

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Abstract

Background. Shiga toxin-induced haemolytic uraemic syndrome (STEC-HUS) is an acute multisystem disorder characterized by renal failure, neurological dysfunction, haemolysis and intravascular thrombosis. Circulating microparticles originating from a number of cell types including thrombocytes and leucocytes are elevated in paediatric patients. In vitro data also suggest modification of leucocyte death by Shiga toxin. Here, we investigated microparticle generation and leucocyte cell death in adult STEC-HUS patients during acute disease and recovery.

Methods. Multi-colour flow cytometry and immunofluorescence were used to assess microparticle concentration and provenience thrombocyte microparticle seeding to leucocytes and leucocyte cell death in adult STEC-HUS patients treated at a tertiary care centre during the STEC-HUS outbreak in Germany in 2011.

Results. Plasma microparticle concentrations of both platelet and leucocyte origin were elevated during acute STEC-HUS. Platelet microparticles (MP) were detected on a high proportion of monocytes and granulocytes. Among therapeutic interventions, plasma exchange reduced platelet marker expression on leucocytes, inhibition of complement had only moderate impact on the number of circulating MP and did not alter platelet microparticle binding to leucocytes. Numbers of apoptotic and necrotic monocytes and granulocytes were significantly increased in patients with STEC-HUS compared to healthy controls. Complement inhibition significantly increased the number of circulating apoptotic cells. Monocyte apoptosis on admission was significantly higher in patients subsequently assigned to plasma exchange or admitted to the intensive care unit.

Conclusions. In STEC-HUS, elevated numbers of circulating MP and dead leucocytes were detected. Monocyte and granulocyte deaths are novel markers of acute STEC-HUS that may actively contribute to tissue destruction by liberation of pro-inflammatory enzymes and cytokines.

Keywords: complement inhibition; leucocyte apoptosis; microparticles; plasma exchange; STEC-HUS

Introduction

The haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) are overlapping syndromes of thrombotic microangiopathies [1, 2]. Typical HUS is a systemic illness induced by enterobacterial Shiga-like toxin (Stx), most commonly produced by enterohaemorrhagic *Escherichia coli* (EHEC) [3]. Clinically, haemolysis and thrombotic microangiopathy are accompanied by varying degrees of renal failure and neurological symptoms ranging from mild confusion to generalized seizures. The uncommon EHEC serotype O104:H4 caused a large outbreak of Shiga toxin-induced haemolytic uraemic syndrome (STEC-HUS) mainly among adults in northern Germany in 2011 [4]. Microparticles (MP) are elevated in a variety of vascular inflammatory conditions [5, 6]. A recent report also describes elevated numbers in children with typical HUS [7]. MP can directly activate leucocytes [8] or transfer activating membrane receptors [9, 10] and other mediators such as microRNA [11, 12]. Complement activation is important in the formation of MP and platelet–leucocyte complexes [13]. Complement was activated in a group of 17 children with diarrhoea-associated HUS [14] and in a mouse model of Stx2-induced HUS [15]. Mechanistically, this can occur directly via the alternative pathway or inhibition of complement regulator factor H [14, 15]. In typical diarrhoea-associated HUS, activated complement compounds were observed on both MP and platelet–leucocyte complexes in a group of 12 children [7]. *In vitro*, a combination of Stx and lipopolysaccharide induced the formation of platelet–leucocyte complexes bearing complement components [7].

Stx is a potent inducer of cell death [16]. It is a bipartite toxin that binds with its B-subunit to the membrane glycolipid globotriaosylceramide (Gb3) that is highly expressed on endothelial cells in the kidney and brain [16]. Stx can also bind to human neutrophilic granulocytes [17–20]. Reports regarding its effect on leucocyte cell death are controversial. It induced apoptosis in the undifferentiated monocyctic cell line THP1 but delayed apoptosis of differentiated, i.e. macrophage-like THP1 [21]. Stx
caused a dose-dependent delay in spontaneous neutrophil apoptosis [22]; however, this was not reproduced in a serum-free system [23]. Data on leucocyte cell death in vivo during Stx-induced TTP-HUS in humans is not currently available.

Little evidence exists for therapeutic interventions in typical STEC-HUS, and even then, most small studies are conducted in children [24, 25]. Plasma exchange was used in severely affected patients during the 1996 Lanarkshire outbreak [26] but no controlled data are available up to date. Eculizumab is a humanized monoclonal antibody against complement protein C5. Eculizumab is the first effective drug therapy for paroxysmal nocturnal haemoglobinuria, a disease characterized by the absence of membrane bound complement inhibiting receptors and the formation of platelet MP [13, 27]. Successful eculizumab treatment of individual patients with congenital HUS has been reported [28–31] and recently, a study of eculizumab in three paediatric patients with diarrhoeal STEC-HUS suggested a beneficial effect on neurological and renal outcome also in this pathophysiological entity [32].

We investigated microparticle generation, leucocyte cell death and impact of treatment with plasma exchange and complement inhibition in a large cohort of adult patients with STEC-HUS at a tertiary care centre.

Materials and methods

Patients and probands

A total of 47 adult patients admitted to Hannover Medical School during the EHEC serotype O104:H4 outbreak in 2011 who consented in writing to donating blood for scientific evaluation were included in this study. Of these patients, (mean age 47 ± 3 years, 68% females), 29 (62%) required haemodialysis (HD) for a mean of 11 ± 6 sessions, 43 (91%) developed neurological symptoms for a mean of 22 ± 3 days, 10 (21%) with local or generalized seizures. Twenty-two (47%) patients were admitted to the intensive care unit and 9 (19%) required mechanical ventilation. Laboratory values at the time of blood draw in patient subgroups are reported in the tables. The protocol was carried out according to the Declaration of Helsinki and approved by the Institutional Review Board at Hannover Medical School. Standard laboratory values were measured at the central clinical laboratory.

Blood collection, microparticle and leucocyte preparation

Blood was drawn into EDTA-coated tubes for leucocyte and citrate tubes (Sarstedt, Nümbrecht, Germany) for microparticle analysis and processed immediately. Whole blood was stained with anti-CD41-PE (RMO52) (Beckman Coulter, Krefeld, Germany), anti-CD16-APC (3G8) (BioLegend, Uithoorn, The Netherlands), anti-CD41-PertCP-Cy5.5 (HPI8) (BD Bioscience, Heidelberg, Germany) and LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit (Molecular Probes, Darmstadt, Germany) followed by erythrocyte lysis with RBC lysis buffer (eBioscience, Frankfurt, Germany) and staining with Annexin V-FITC (BD Bioscience) according to the manufacturer’s instructions.

For microparticle isolation, platelet-free plasma was obtained by centrifugation at 5000 g for 5 min at 20°C twice, and MP were concentrated by centrifugation at 17 000 g for 10 min at 4°C, resuspended in Annexin V-binding buffer and stained with Annexin V-FITC (BD Bioscience) and antibodies as indicated. All buffers were filtered using a 0.2-μm filter (Sartorius Stedim Biotech GmbH, Göttingen, Germany) before use. The number of MP per microlitre of plasma was calculated using Trucount Tubes (BD Biosciences) according to the manufacturer’s instructions.

Flow cytometry analysis

Flow cytometry was performed on a FACSCanto (BD Biosciences) with FACSDiva Software version 6.0. Data were analysed using FlowJo software (Tree Star, Ashland, OR). MP were gated using Megamix beads (Biocytex Marseille, France) by SSC-A-AnnexinV, identifying MP as Annexin V positive particles below 1 μm size with microparticle scatter properties.

Immunofluorescence staining

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood of patients with STEC-HUS by Ficoll–Paque gradient centrifugation. After erythrocyte lysis, 10^7 PBMCs were fixed with 100% acetone for 10 min. After 30-min blocking with 1% bovine serum albumin, the slides were stained with anti-CD41 (HIP8) or anti-CD3 (HI3a) (both from BD Bioscience) overnight at 4°C. Cy3 donkey antimouse (Jackson ImmunoResearch, Newmarket, UK) was used as secondary antibody, and mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Dianova, Hamburg, Germany) was applied. Fluorescence images were obtained with a Zeiss Axiosplan-2 imaging microscope using AxiosVision 4.6 (Zeiss, Jena, Germany).

Statistical analysis

Two-tailed student t-test or, if more than two groups were compared, analysis of variance with appropriate post hoc test was used and this is indicated in the figure legends; P-values <0.05 were considered significant. Data are expressed as mean ± SEM. P-values are indicated with *P < 0.05, **P < 0.01 and ***P < 0.001.

Results

STEC-HUS increases peripheral blood microparticle concentration

Recent data have shown elevated microparticle numbers in children with typical HUS [7, 33]. We therefore investigated whether this also applies to the adult form of STEC-HUS caused by serotype O104:H4 [4]. Microparticle concentrations were determined in platelet poor plasma processed immediately after blood draw from patients with acute STEC-HUS (on admission, 0–5 days after STEC-HUS onset, before therapy) and healthy controls (Table 1). MP were determined by size and Annexin V positivity (Figure 1A). Circulating microparticle concentrations were significantly elevated in patients with acute STEC-HUS (Figure 1B).

To determine the origin of MP, we employed staining for CD41 as a platelet marker and CD14 and CD16 as monococyte and granulocyte markers. MP of all origins were significantly elevated in patient plasma (Figure 1C–E).

Table 1. Characteristics of patients with STEC-HUS and healthy controls

<table>
<thead>
<tr>
<th>Healthy controls</th>
<th>STEC-HUS Day 0–5 (MP analysis)</th>
<th>STEC-HUS Day 0–5</th>
<th>STEC-HUS Day 25–35</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (Male)</td>
<td>11 (2)</td>
<td>5 (1)</td>
<td>30 (9)</td>
</tr>
<tr>
<td>Age</td>
<td>33 ± 2</td>
<td>43 ± 8</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>Hb (mg/dL)</td>
<td>10.4 ± 1.2</td>
<td>9.9 ± 0.3</td>
<td>9.5 ± 0.4</td>
</tr>
<tr>
<td>Thrombocytes (10^5/μL)</td>
<td>136 ± 38</td>
<td>83 ± 12</td>
<td>229 ± 23</td>
</tr>
<tr>
<td>Leucocytes (10^3/μL)</td>
<td>14 ± 6</td>
<td>14 ± 2</td>
<td>4.5 ± 0</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>885 ± 497</td>
<td>925 ± 126</td>
<td>245 ± 18</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>101 ± 16</td>
<td>272 ± 39</td>
<td>103 ± 9</td>
</tr>
</tbody>
</table>

*Values are given as mean ± SEM.
Seeding of platelet markers on peripheral blood leucocytes in STEC-HUS

Platelet MP and dust can adhere to peripheral blood leucocytes in STEC-HUS. (A, B) Circulating MP were determined as Annexin V+ events <1 μm of size as described in Materials and methods. In patients with acute STEC-HUS (before therapy, n = 5), counts were significantly elevated compared to healthy controls (n = 11). [(A), typical example and (B) means ± SEM]. (C–E) Cell provenience of MP was determined by staining with anti-CD41 (platelets, C), and anti-CD14 (D) and CD16 (E) (monocytes, granulocytes) (means ± SEM of n = 5 untreated patients and n = 11 controls). P-values are indicated with *P < 0.05, **P < 0.01 and ***P < 0.001.

Complement has been found on platelet MP in paediatric patients [7, 33]. Case reports suggesting a beneficial effect of complement inhibition STEC-HUS [32] have prompted the use of eculizumab during the investigated outbreak. In our centre, eculizumab was given to patients with high haemolytic activity and/or neurological symptoms after three courses of plasma exchange (Table 2). To test the effect of complement inhibition on microparticle generation in this STEC-HUS setting, we measured MP before and after application of eculizumab. MP have a half-life of a few hours in vivo [38]. Administration of eculizumab significantly decreased numbers of circulating MP (Figure 3D–G), but their concentration was still significantly higher than in healthy individuals (Figure 1). Decreases in platelet and leucocyte marker-positive microparticle subpopulations did not reach significance; only CD16+ microparticle concentration was reduced significantly (Figure 3F). HD treatment is known to increase circulating microparticle concentrations [5]. We therefore analysed whether or not microparticle concentrations were different in HD-treated patients. Indeed, patients receiving HD tended to have higher plasma MP concentrations at start of eculizumab therapy (740 ± 127 MP/μL, n = 7 versus 452 ± 64 MP/μL, n = 8, P = 0.05). On Day 2 after eculizumab, this difference disappeared owing to a more marked decrease in the subgroup of patients requiring HD (380 ± 68 MP/μL in HD versus 393 ± 86 MP/μL).

To investigate whether the moderate decrease in microparticles affected platelet marker seeding on peripheral leucocytes, the proportions of CD41+ monocytes (Figure 3G) and granulocytes (Figure 3H) were determined by flow cytometry. Values before starting eculizumab after three courses of plasma exchange were significantly lower than values obtained on Day 0–5 after onset of STEC-HUS, consistent with the decrease observed in patients undergoing a single round of plasma exchange (Figure 3A, B). No further decrease was observed during eculizumab therapy (Figure 3G, H). These data suggest that mechanisms other than complement activation play a role in platelet marker seeding on leucocytes in STEC-HUS.

STEC-HUS increases circulating apoptotic and necrotic leucocyte numbers

Shiga toxin can alter leucocyte death [16]. Leucocyte death has to the best of our knowledge not previously been assessed in patients with typical STEC-HUS. We therefore investigated whether altered numbers of circulating apoptotic or necrotic leucocytes can be detected during acute STEC-HUS in vivo. In both the monocytic and granulocytic compartments, the proportions of apoptotic cells as determined by AnnexinV staining...
were significantly elevated when compared to healthy controls and patients during recovery (Figure 4). The proportion of necrotic cells showed a similar course. These data suggest increased leucocyte cell death during STEC-HUS in vivo.

After the first course of plasma exchange, there was a tendency toward a decrease in circulating apoptotic leucocyte (Figure 5A, C). Their proportion was decreased significantly after three exchange cycles at the start of eculizumab therapy (Figure 5B and D). Effects on
necrosis had the same tendency but did not reach significance (data not shown). However, the proportion of circulating apoptotic monocytes and granulocytes significantly increased after complement inhibition by eculizumab (Figure 5B and D).

We tested whether or not this novel parameter correlated with course of the disease. The numbers of apoptotic monocytes on Day 0–5 of STEC-HUS were significantly higher in patients subsequently assigned to receive plasma exchange (Figure 5E) by the criteria detailed above or admitted to the intensive care unit (Figure 5F). This suggests monocyte apoptosis as a possible predictor of disease severity in this cohort of severely affected patients.

**Discussion**

Shiga toxins damage multiple cell types and cause severe systemic illness. Our data show increased microparticle generation and leucocyte death in STEC-HUS in vivo.
MP and leucocyte death in STEC-HUS

MP are generated from various cell types and have been implicated in the pathophysiology of a large number of diseases. Similarly to previous reports, where STEC-HUS is caused by the classic serotype O157:H7 in the paediatric population [7, 33], we found markedly increased numbers in adult patients infected with serotype O104:H4. Also, we observed binding of platelet marker to circulating monocytes, and to a somewhat lesser degree granulocytes. It remains to be investigated whether MP are mediators in addition to markers of disease activity in STEC-HUS. It has to be noted in caution that while circulating microparticle numbers were clearly higher in patients than controls, neither microparticle numbers nor adhesion to circulating leucocytes correlated with prognosis (data not shown). Relatively small patient numbers in a homogenous cohort with manifest STEC-HUS transferred to a tertiary care centre and subsequent treatment are possible explanations for this finding unrelated to biologic microparticle effects. Platelet MP are well known to transfer receptors [9, 10] and microRNA [12], and possibly also exogenous substances such as Stx [17] to leucocytes. Whether or not microparticle concentration during the diarrhoeal phase of EHEC infection predicts onset of STEC-HUS remains to be studied.

To better characterize the microparticle dynamics in STEC-HUS, we also studied treatment effects on microparticle generation and adhesion of platelet MP to leucocytes. It has been reported that complement activation by Stx induced microparticle binding to monocytes and granulocytes in vitro [7, 33] and complement C3 bound to platelet–monocyte complexes in vivo [7]. However, in our cohort of adult patients, while plasma exchange decreased the proportion of...
leucocytes carrying platelet MP, complement C5 inhibition in vivo did not alter the number of platelet marker-positive leucocytes and only moderately decreased circulating microparticle concentration. In fact, C3a activation upstream of C5 has been implicated in Stx-mediated toxicity in a model of murine HUS [39]. Here, renal microvascular thrombus formation was inhibited by blockade of the C3a receptor. This pathway would remain unaffected by blocking C5 downstream in the complement cascade. Also, in a group of 17 children with diarrhoea-associated HUS terminal complement complex C5b-9 formation was increased but did not correlate with renal injury or other complications [14]. This would be consistent with a model where in contrast to atypical HUS, activation of the terminal C5b-9 was not of central importance for development of the disease.

In vitro findings regarding the effects of Stx on cell death in a monocytic cell line and isolated granulocytes are diverse and partially contradictory [16]. Here, we show in a large cohort of adult STEC-HUS patients that the numbers of circulating necrotic and apoptotic monocytes and granulocytes were significantly increased in vivo. Innate leucocytes have a comparatively short life span and especially neutrophilic granulocytes possess a strong constitutional apoptosis program. Therefore, increased numbers of dead cells may be both due to increased rates of cell death and defective clearance mechanisms [16]. The numbers of circulating dead leucocytes were significantly lower after three courses of plasma exchange. Reduction of Stx concentrations in plasma but also retention of pre-activated and/or dead cells in the extracorporeal circulation are possible explanations of this finding. In contrast to this and our initial hypothesis of a role of pore complex activation in inducing leucocyte death, the proportion of circulating dead cells even significantly increased early after application of eculizumab in vivo. Whether this is due to a direct eculizumab effect or rather that complement was required to clear leucocytes killed by complement independent mechanisms such as direct Stx toxicity remains to be investigated.

Innate leucocytes, especially neutrophilic granulocytes, contain large amounts of proteolytic and pro-inflammatory mediators in pre-formed granules, which can, if not properly disposed of during constitutive apoptosis but rather liberates during death of activated cells, cause significant inflammatory responses [40]. In addition, in STEC-HUS, Stx transfer by leucocytes to the endothelium can induce endothelial activation and cell death [18]. Our finding of increased leucocyte death prompted us to investigate whether its rate on admission predicted the severity of the disease. In fact, rates of apoptotic monocytes in the circulation correlated with later assignment to plasma exchange and admission to the intensive care unit, both measures of disease severity. While waiting to be repeated in additional cohorts, this novel finding is consistent with the hypothesis that the increase in leucocyte death contributes to systemic disease in STEC-HUS.

In summary, our data provides a detailed description of microparticle number, subtypes and leucocyte cell death in STEC-HUS. Monocyte apoptosis is a possible new marker of disease severity.

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References


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