Microparticles, coagulation and inflammation

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Chapter 5

Microparticles from patients with multiple organ dysfunction syndrome and sepsis support coagulation through multiple mechanisms


ABSTRACT

Aim. We investigated the occurrence and thrombin-generating mechanisms of circulating microparticles in patients with multiple organ dysfunction syndrome (MODS) and sepsis.

Methods. Microparticles, isolated from blood of patients (n = 9) and healthy controls (n = 14), were stained with cell-specific monoclonal antibodies (MoAbs) or anti-tissue factor (anti-TF) MoAb and annexin V, and analyzed by flow cytometry. To assess their thrombin-generating capacity, microparticles were reconstituted in normal plasma. The coagulation activation status in vivo was quantified by plasma prothrombin fragment 1+2 ($F_{1+2}$) and thrombin-antithrombin (TAT) measurements.

Results. Annexin V-positive microparticles in the patients originated predominantly from platelets (PMP), and to a lesser extent from erythrocytes, endothelial cells (EMP) and granulocytes (GMP). Compared to healthy controls, the numbers of annexin V-positive PMP and TF-exposing microparticles were decreased ($P = < 0.001$ for both), EMP were decreased (E-selectin, $P = 0.003$) or found equal (CD144, $P = 0.063$), erythrocyte-derived microparticles were equal ($P = 0.726$), and GMP were increased ($P = 0.008$). GMP numbers correlated with plasma concentrations of elastase ($r = 0.70$, $P = 0.036$), but not with C-reactive-protein or interleukin-6 concentrations. Patient samples also contained reduced numbers of annexin V-negative PMP, and increased numbers of erythrocyte-derived microparticles and GMP ($P = 0.005$, $P = 0.021$ and $P < 0.001$, respectively). Patient microparticles triggered thrombin formation, which was reduced compared to the healthy controls ($P = 0.008$) and strongly inhibited by an anti-factor XII MoAb (two patients), by anti-factor XI MoAb (eight patients) or by anti-TF MoAb (four patients). Concentrations of $F_{1+2}$ and TAT were elevated ($P = 0.005$ and $P = 0.001$, respectively) and correlated inversely with the number of circulating microparticles ($r = -0.51$, $P = 0.013$, and $r = -0.65$, $P = 0.001$, respectively) and their thrombin generation capacity ($F_{1+2}$: $r = -0.62$, $P = 0.013$).

Conclusions. In patients with MODS and sepsis relatively low numbers of microparticles are present that differ from controls in their cellular origin, numbers and coagulation activation mechanisms.
INTRODUCTION

Cells undergoing activation or apoptosis release small parts of their outer membrane, the so-called microparticles. Extensive studies have been reported on microparticles generated from blood platelets (PMP) in vitro [6,49]. These microparticles expose negatively charged phospholipids, thereby providing binding sites for activated coagulation factors V (factor Va), VIIIa, IXa and XIa [6,43,46,49]. As a consequence, these PMP are procoagulant by providing "phospholipid cofactor". In vitro, other cell-types such as monocytes, endothelial cells and erythrocytes also release microparticles upon appropriate stimulation [51,53,58].

In vivo, increased numbers of PMP are found in the circulation of patients with an increased risk for thromboembolic events, i.e. patients with diabetes [14,15], patients undergoing cardiac surgery [138], or patients suffering from acute coronary ischaemia [12], heparin-induced thrombocytopenia [17], myocardial infarction [22], uremia [23], idiopathic thrombocytopenic purpura [24] and disseminated intravascular coagulation (DIC) [29,31]. Previously, we reported elevated numbers of PMP and erythrocyte-derived microparticles in the pericardial fluid of patients undergoing CPB surgery, and demonstrated that the isolated microparticles facilitated thrombin generation via the extrinsic pathway, i.e. tissue factor (TF)/factor VII-dependent [33]. Subsequently, we reported elevated numbers of PMP and microparticles derived from monocytes and granulocytes in the circulation of patients with meningococcal septic shock, and provided evidence that exposure of TF on microparticles might be involved in the pathogenesis of DIC [29]. Finally, we were also able to measure low numbers of microparticles -especially from platelets and erythrocytes- in the circulation of healthy individuals. Those microparticles triggered low levels of thrombin generation in vitro via a TF/factor VII-independent mechanism. This thrombin generation was partially inhibited by blockade of factors XII or XI [139]. Recently, Combes and coworkers reported elevated numbers of endothelial cell-derived microparticles (EMP) in the circulation of patients with systemic lupus erythematosus when compared to healthy individuals [56].

Meningococcal septic shock is a very severe, rapidly progressive disease, which may not be representative for other more protracted septic conditions. In the present
study we investigated the cellular source and thrombin-generating capacity of microparticles in patients with multiple organ dysfunction syndrome (MODS) and sepsis, and a possible relation of those microparticles to the activation status of the coagulation system in vivo. We also investigated a possible relation between the number of microparticles in the circulation and the extent of the inflammatory response, as indicated by the plasma concentrations of interleukin-6 (IL-6), elastase and the acute phase reactant C-reactive protein (CRP).

PATIENTS, MATERIALS AND METHODS

Patients and healthy individuals

Patients with MODS and sepsis were investigated. The patients entered the study between July 1998 and March 1999 and were hospitalized in the Intensive Care Unit of the Leiden University Medical Center (LUMC). Of the nine patients (2 female and 7 male; age: 60 years [median], range 26 - 71), six had bacteremia whereas the others had a local bacterial infection. Patients developed MODS and sepsis after surgery (six patients), pancreatitis (one), multitrauma (one) or E. coli meningitis (one). Patients were considered to have sepsis if they fulfilled the criteria for a systemic inflammatory response syndrome (SIRS) and had evidence for a systemic and/or local bacterial infection [140]. The criteria to establish SIRS were more than one of the following: (I) a temperature > 38 °C or < 36 °C; (II) tachycardia (> 90 beats/minute); (III) tachypnea (respiratory rate > 20 breaths/minute or PaCO₂ < 32 mm Hg); and (IV) a white blood cell count > 12 x 10⁹/L or < 4 x 10⁹/L, or the presence of > 10% immature neutrophils [140]. The diagnosis SIRS was made by the attending physician and checked by one of the study clinicians (JB). MODS was defined as a failure of 3 or more organ systems for at least 24 hours [140]. Of the nine patients, five patients died within 28 days. Individual patient characteristics, including platelet- and white blood cell counts, are presented in Table 1. Of the nine patients, 7 had elevated numbers of white blood cell counts (> 10 x 10⁹/L), whereas 5 patients were thrombocytopenic (< 150 x 10⁹/L). As controls, 14 adult healthy individuals were also investigated for the presence, cellular source and thrombin-generating capacity of microparticles. The healthy individuals had not taken any medication for at least ten days prior to the blood collection. The
study was approved by the medical ethical committee (234-94/5/DPE/EN) of the LUMC.

Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Gender</th>
<th>Platelet count</th>
<th>WBC count</th>
<th>Cause of sepsis</th>
<th>Positive culture</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67</td>
<td>M</td>
<td>54</td>
<td>14.3</td>
<td>Post-operative</td>
<td>Blood</td>
<td>Survivor</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>M</td>
<td>193</td>
<td>15.8</td>
<td>Post-operative</td>
<td>Blood</td>
<td>Survivor</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>M</td>
<td>102</td>
<td>6.0</td>
<td>Post-operative</td>
<td>Blood</td>
<td>Non-survivor</td>
</tr>
<tr>
<td>4</td>
<td>43</td>
<td>F</td>
<td>223</td>
<td>25.3</td>
<td>Post-operative</td>
<td>Blood</td>
<td>Non-survivor</td>
</tr>
<tr>
<td>5</td>
<td>71</td>
<td>M</td>
<td>113</td>
<td>29.6</td>
<td>Pancreatitis</td>
<td>Blood</td>
<td>Survivor</td>
</tr>
<tr>
<td>6</td>
<td>61</td>
<td>F</td>
<td>63</td>
<td>9.3</td>
<td>E. coli meningitis</td>
<td>CSF</td>
<td>Non-survivor</td>
</tr>
<tr>
<td>7</td>
<td>26</td>
<td>M</td>
<td>167</td>
<td>18.1</td>
<td>Multi trauma</td>
<td>Blood</td>
<td>Non-survivor</td>
</tr>
<tr>
<td>8</td>
<td>60</td>
<td>M</td>
<td>166</td>
<td>15.4</td>
<td>Post-operative</td>
<td>Sputum</td>
<td>Non-survivor</td>
</tr>
<tr>
<td>9</td>
<td>62</td>
<td>M</td>
<td>121</td>
<td>13.9</td>
<td>Post-operative</td>
<td>Aneurysm</td>
<td>Survivor</td>
</tr>
</tbody>
</table>

1M=ma/e, F=female; 2Whole blood platelet- and white blood cell counts (x 10⁰/L); 3Within 28 days after onset of sepsis; 4Cerebrospinal fluid.

Collection of blood samples

Blood was collected into 3.2% trisodium citrate (BD, San Jose, CA, USA). Blood cells were removed within 5 minutes after blood collection by centrifugation for 20 minutes at 1550g and room temperature. For flow cytometry, microparticles were isolated (see below) from fresh plasma samples. The concentrations of prothrombin
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fragment 1+2 (F₈₋₂), thrombin-antithrombin complex (TAT), IL-6, elastase and CRP, and thrombin generation experiments were determined in aliquots of plasma that were first snap frozen in liquid nitrogen, and then stored at -80 °C until use.

**Reagents and assays**

Reptilase was obtained from Roche (Basel, Switzerland) and the chromogenic substrate S2238 from Chromogenix AB (Mölndal, Sweden). Murine normal serum was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB; Amsterdam, The Netherlands), anti-Glycoporin A-fluorescein isothiocyanate (FITC; JC159, immunoglobulin (Ig)G₁) and CD61-FITC (Y2/51, IgG₁) from Dako A/S (Glostrup, Denmark), IgG₁-FITC (X40) from BD (San Jose, CA, USA), CD62E-FITC (1.2B6, IgG) from Serotec Ltd (Oxford, England), CD66b-FITC (80H3, IgG₁k) from Coulter/Immunotech (Marseille, France), CD144-FITC (BMS158Fl, IgG₁) from MedSystems Diagnostics GmbH (Vienna, Austria), annexin V-allophycocyanine (APC) from Caltag Laboratories (Burlingame, CA, USA) and annexin V-PE from PharMingen (San Jose, CA, USA). OT-2 (0.71 mg/mL), a MoAb which inhibits the activity of factor XII(a), was prepared as described earlier [85]. MoAb directed against factor XI (clone XI-1 (0.92 mg/mL)) was also from the CLB. Anti-TF-FITC (4508CJ, IgG₁) and polyclonal rabbit anti-human TF (1 mg/mL; clone 4502) were from American Diagnostics, Inc. (Greenwich, CT, USA). Plasma concentrations of F₁₋₂ and TAT (Behring Diagnostics GmbH, Marburg, Germany), elastase (DPC, Nauheim, Germany) and IL-6 (CLB) were determined by ELISA as described by the manufacturers. CRP was measured on a Hitachi 911 analyzer (Roche, Basel, Switzerland) by immunoturbidimetric assay as described by the manufacturer.

**Isolation of microparticles**

After removal of cells, 250 μL plasma were centrifuged for 30 minutes at 17570g and 20 °C. Subsequently, 225 μL of (microparticle-free) plasma were removed. The remaining (microparticle-enriched) plasma, 25 μL, was diluted with 225 μL of phosphate-buffered saline (PBS; 154 mmol/L NaCl, 1.4 mmol/L phosphate, pH 7.4), containing 10.9 mmol/L trisodium citrate to prevent coagulation activation.
Microparticles from patients with MODS and sepsis

Microparticles were resuspended and centrifuged for 30 minutes at 17570g at 20 °C. Again, 225 μL of the supernatant were removed and microparticles were resuspended in the remaining 25 μL. For the thrombin generation experiments, 20 μL of this suspension were used. For flow cytometry, 25 μL microparticles suspension was diluted fourfold with PBS/citrate buffer, of which 5 μL were used per incubation with MoAb and annexin V.

Flow cytometric analysis

Microparticle analysis was performed as described previously [29,33]. Briefly, microparticles (5 μL) were diluted in 35 μL PBS containing 2.5 mmol/L CaCl₂ (pH 7.4) and 5 μL of 1 to 500 diluted (in PBS) normal mouse serum. After incubation for 15 minutes at room temperature, 5 μL annexin V-PE and 5 μL FITC-labeled cellspecific MoAbs or isotype-matched control antibody were added. The mixtures were incubated in the dark for 15 minutes at room temperature. Subsequently, 200 μL PBS/calcium buffer were added and the suspensions centrifuged for 30 minutes at 17570g and 20 °C. Finally, 200 μL of (microparticle-free) suspension were removed. The microparticles were resuspended with 300 μL PBS/calcium buffer before flow cytometry. All samples were analyzed for 1 minute during which the flow cytometer analyzed approximately 150 μL of the suspension. To estimate the number of microparticles /L plasma, the number of microparticles (N) found in the upper right (marker positive and annexin V positive) and lower right (marker positive and annexin V negative) quadrants of the flow cytometry analysis (FL1 versus FL2, corrected for isotype control and autofluorescence) was used in the formula: Number/L = N x [100/5] x [355/150] x [10⁶/250]. The lower detection limit of the particle count was established in the samples with the IgG control as 5 x 10⁶ microparticles/L. The samples were analyzed in a FACScan flow cytometer with CellQuest software (BD, San Jose, CA, USA). Forward scatter (FSC) and side scatter (SSC) were set at logarithmic gain. To distinguish microparticles from events due to noise, microparticles were identified on FSC, SSC and binding of a MoAb directed against a cell-specific antigen. To identify annexin V-positive events, a threshold was placed in a microparticle sample prepared without any additions to correct for autofluorescence.
To identify microparticles that bound cell-specific MoAbs, microparticles were incubated with identical concentrations of isotype-matched control antibodies to set the threshold. FITC-fluorescence was measured in the FL1 channel and PE-fluorescence in the FL2 channel. The anti-CD144-antibody became only recently available to us, once we had started to perform our analysis on a FACSCalibur flow cytometer from Becton Dickinson (San Jose, CA, USA). This antibody was used in conjunction with annexin V-APC and anti-CD62E-PE. In all other aspects the CD144 analysis was performed as described for the other antibodies.

**Thrombin generation assay**

The thrombin generation test (TGT) as described by Béguin et al [127] was used to assess the thrombin-generating capacity of the microparticles. In brief, isolated microparticles were reconstituted in defibrinated, microparticle-free normal plasma. Microparticles were isolated from stored (-80 °C) plasma from patients (n = 9) and healthy controls (n = 6). Defibrinated plasma was prepared by incubating microparticle-free normal plasma (a pooled plasma from 20 healthy individuals, that had been centrifuged for 30 minutes at 17570g and 20 °C) with reptilase for 10 minutes at 37 °C and, subsequently, for 10 minutes on melting ice. The fibrin clot was removed by centrifugation for 30 minutes at 17570g and 20 °C. Since microparticles adhere to fibrin, the plasma aliquots from which microparticles were isolated were not defibrinated [87]. Microparticles (20 μL) were added to 120 μL of defibrinated plasma in all experiments. At t = 0, thrombin generation was triggered by the addition of 30 μL CaCl₂ (16.7 mmol/L final concentration) to a prewarmed (37 °C) mixture of plasma, microparticles and buffer A (10 μL; 50 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.05% bovine serum albumin, pH 7.35). After t=0, at fixed intervals, 3 μL aliquots were removed from this mixture and added to 147 μL prewarmed (37 °C) chromogenic substrate S2238 in buffer B (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 20 mmol/L EDTA and 0.05% bovine serum albumin, pH 7.90). After 3 minutes, the conversion of S2238 was stopped by the addition of 90 μL citric acid (1 mol/L) and the generated amount of p-nitroaniline was determined at λ = 405 nm. In the inhibition experiments, the mixture of plasma plus buffer A, and separately the microparticles,
Microparticles from patients with MODS and sepsis

were incubated with 20 μL and 10 μL of antibodies, respectively. In preliminary experiments the concentration yielding maximal inhibition was determined for each antibody. These were anti-TF (1 mg/mL initial concentration), anti-FXI (0.92 mg/mL) and anti-FXII (0.71 mg/mL). After 30 minutes pre-incubation at room temperature, the microparticles were added and incubated for 10 minutes at 37 °C, whereupon the thrombin generation was started by addition of CaCl₂. For quantitative analysis, the results were determined as the area under the curve and expressed as mg of the graph paper being weighed. Compared to unfrozen microparticles from healthy controls, after freeze/thawing these microparticles generate more thrombin as the area under the (thrombin generation) curves increases about two fold, but the proportion of inhibition by individual antibodies (such as anti-FXI, anti-FXII, anti-TF or anti-FVII) is unaffected by freeze/thawing. In preliminary experiments, we observed that the increase in the area under the curve of the thrombin generation experiments upon freezing/thawing may be somewhat dependent upon the composition of the microparticles in the plasma sample. This indicates that the absolute thrombin generation capacities of stored microparticle preparations have to be interpreted with some caution. However, the freezing/thawing did not influence the percentage of inhibition by the various antibodies regardless of the composition of the microparticle populations, and thus the pathways observed to initiate the thrombin generation seem to be unaffected by the freezing/thawing process [139].

Statistical methods

Data were analyzed with SPSS for Windows, release 9.0. The data obtained in the healthy individuals were log transformed to calculate the 2.5 and 97.5 percentiles of the reference range. Dependent upon the parameter under investigation, individual patient values either below or above those percentiles are indicated. Differences in microparticle numbers between the groups of patients and healthy individuals were tested with the Mann Whitney U test. Correlations were determined with the Spearman’s Rho test.
RESULTS

Number and cellular origin of microparticles in patients with MODS and sepsis

A representative flow cytometric analysis of microparticles circulating in a patient is presented in Figure 1. The patient plasma mainly contained platelet- and erythrocyte-derived microparticles, but also microparticles from endothelial cells (EMP) and granulocytes (GMP). For the detection of EMP, antibodies directed against E-selectin and VE-Cadherin (CD62E and CD144, respectively) were used. Also TF (CD142) was measured. A similar analysis of a healthy individual is presented in Figure 2. Here, the absence of GMP and the higher number of PMP are noteworthy. Remarkably, a larger number of EMP were measured with the anti-CD62E antibody as compared to the anti-CD144 antibody in both patients and healthy controls (Figures 1 and 2, respectively). Quantitative data are presented in Table 2, in which not only microparticles numbers of the marker- or TF-positive and annexin V-positive populations are summarized (upper right quadrants of Figures 1 and 2), but also annexin V-negative events that were marker- or TF-positive (lower right quadrants of Figures 1 and 2). On average, the numbers of annexin V-positive microparticles of platelet-origin were decreased and those of endothelial cell-origin either decreased (CD62E) or similar (CD144). The numbers of erythrocyte-derived microparticles were similar and the numbers of GMP were elevated. The numbers of annexin V-negative PMP were decreased, EMP were comparable and the numbers of erythrocyte-derived microparticles and GMP were increased. Figure 3 shows the numbers of PMP, erythrocyte-derived microparticles, EMP and GMP in the individual patients. The number of annexin V- and TF-positive microparticles, not specified per cell type, was reduced in the patient group, but numbers of annexin V-negative and TF-positive microparticles were equal for this antigen. There was a considerable overlap in the ranges of the number of microparticles between the healthy volunteers and the patients, but, dependent upon the cellular origin of the microparticles, 0 to 8 of the patients had numbers of microparticles below the 2.5 or above the 97.5 percentile of the range in the healthy controls (Table 2). Microparticles of T helper cells (CD4), T suppressor cells (CD8), B-cells (CD20) and plasma cells (CD38) were below the detection limit in both patients and controls (data not shown).
Figure 1. Representative dot plots of microparticles (MP) in plasma of a patient with MODS and sepsis. Microparticles were isolated, double labeled with annexin V (PE-labeled) and a MoAb directed against a specific cell marker or TF (both FITC-labeled), and analyzed by flow cytometry as described in the Patients and Methods section. The binding of annexin V is indicated on the y-axis, and the expression of the cell marker on the x-axis. The fluorescence thresholds were set as described in the Patients and Methods section, i.e., with a isotype-identical control antibody for the cell marker and in the absence of annexin V for annexin V binding. Data of patient 7 are presented.
Figure 2. Representative dot plots of microparticles in plasma of a healthy individual. For details see legend Figure 1.
Microparticles from patients with MODS and sepsis

Table 2. Numbers and cellular origin of circulating microparticles in patients with MODS and sepsis (n = 9) and healthy individuals (n = 14).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Patients</th>
<th>Controls</th>
<th>N₂/N₁ (^3)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets</td>
<td>CD61</td>
<td>114 (65 - 208)(^1)</td>
<td>3/9 (lower)</td>
<td>0.001(^1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 (17 - 185)(^2)</td>
<td>2/9 (lower)</td>
<td>0.005(^2)</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Glyco A</td>
<td>24 (&lt; 5 - 165)</td>
<td>4/9 (higher)</td>
<td>0.174</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94 (26 - 293)</td>
<td>4/9 (higher)</td>
<td>0.021</td>
</tr>
<tr>
<td>Endothelial</td>
<td>CD62E</td>
<td>93 (57 - 309)</td>
<td>2/9 (lower)</td>
<td>0.010</td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td>17 (8 - 66)</td>
<td>1/9 (higher)</td>
<td>0.238</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>CD66b</td>
<td>12 (&lt; 5 - 145)</td>
<td>5/9 (higher)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43 (&lt; 5 - 110)</td>
<td>8/9 (higher)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(TF-positive)</td>
<td>(CD142)</td>
<td>14 (5 - 30)</td>
<td>4/9 (lower)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16 (&lt; 5 - 36)</td>
<td>1/9 (lower)</td>
<td>0.238</td>
</tr>
</tbody>
</table>

\(^1\)Cell-marker- or TF-positive and annexin V-positive microparticles (x 10⁵/L); \(^2\)Cell-marker- or TF positive and annexin V-negative microparticles (x 10⁶/L); \(^3\)N₁: total number of patient samples tested, N₂: number of patient samples with microparticle numbers below the 2.5 or above the 97.5 percentile of the reference range in the healthy controls.
Figure 3. Microparticle numbers in the individual patients. Numbers of platelet-, erythrocyte-, endothelial-, granulocyte-derived microparticles are shown as well as the number of TF-positive microparticles \((x 10^6/L \text{ plasma})\). For each individual patient the numbers of marker- and annexin V-positive (light gray bars) as well as marker-positive but annexin V-negative microparticles (hatched bars) are shown. The data in the healthy controls are depicted as median with range.
Thrombin-generating capacity, initiation pathways and relation to in vivo coagulation activation status

Figure 4 shows thrombin generation curves obtained with microparticles from two patients and a representative healthy individual in the absence and presence of inhibitory MoAb. The curves from patients 1 and 7 are provided, because patient 1 demonstrated extreme inhibition with anti-factor XII, in contrast to patient 7.

Figure 4. Thrombin generation by isolated microparticles from two patients and from a representative healthy individual in defibrinated normal plasma. Thrombin generation was performed in the absence (■) or presence of antibodies to TF (□), factor XII (●) or factor XI (○), as described in Patients and Methods. The background, i.e. recalcified microparticle-free plasma, is depicted by (◇).

Overall data are provided in Figure 5. As represented in this figure, the thrombin-generating capacity, expressed as the area under the curve in the assay, varied widely between the patients but was overall reduced compared to the controls ($P = 0.008$).
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Thrombin generation by microparticles from healthy individuals was on average not inhibited by anti-TF, slightly inhibited by anti-factor XII (on average 12%) and by anti-factor XI (36%). Three patients had a significantly reduced thrombin generation capacity of the microparticles; four had an increased inhibition by anti-TF, two by anti-factor XII and all but one of the nine with anti-factor XI (Figure 5).

Figure 5. The effect of inhibitory antibodies on thrombin generation by microparticles from patients and from healthy individuals. Median and range of the data in the healthy individuals (n = 6) are presented, together with the individual data of patients 1-9. Note that the data in the upper left figure represent absolute values of thrombin generation, expressed as the area under the curve. Those in the other figures are relative values, i.e. the percentage remaining thrombin generation in the presence of the antibody compared to the uninhibited value in that patient. The * indicates findings lower than the 2.5 percentile of the reference range in the healthy controls.
In vivo coagulation activation status and relation to the number of circulating microparticles

Concentrations of both $F_{1+2}$ and TAT were determined to assess the activation status of the coagulation system in vivo. As shown in Figure 6A, concentrations of both $F_{1+2}$ and TAT were significantly elevated in the patients compared to the healthy controls ($F_{1+2}$ in patients 1.20 [median], range 0.46 - 3.48 and controls 0.59 [0.32 - 1.91], $P = 0.005$; TAT in patients 5.50 [2.20 - 14.70] and controls 1.04 [0 - 3.63], $P = 0.001$), indicating that the coagulation system in the patients was activated.

**Figure 6.** Relationship between in vivo thrombin generation and number and thrombin-generating capacity of circulating microparticles. A. Median and range of concentrations of $F_{1+2}$ (gray bars) and TAT (hatched bars) from patients ($n = 9$) and controls ($C; n = 14$). B. Correlation between the number of circulating, annexin V-positive microparticles and plasma TAT-concentrations. C. Correlation between the number of circulating microparticles and the plasma $F_{1+2}$ concentrations. D. Correlation between the extent of thrombin generation by the microparticles and plasma concentrations of $F_{1+2}$. Patients are depicted as ○ and controls as ● in Figures 5B-D. *$P < 0.05$ and **$P < 0.001$ are indicated in Figure 5A. Spearman’s rank correlation analysis is provided in Figures 5B-D. Two of the controls are not depicted in Figure 5B, because their plasma concentration of TAT was below 0.1 μg/L.
Next, we determined correlations between the in vivo activation status of the coagulation system and the number of circulating microparticles as well as their thrombin-generating potential in plasma. As shown in Figure 6B and 6C, the numbers of circulating, annexin V-binding microparticles in patients and controls inversely correlated with the concentrations of both $F_{1+2}$ and TAT ($r = -0.51, P = 0.013$, and $r = -0.65, P = 0.001$, respectively). A negative correlation trend was present between the thrombin generation capacity (area under curve) and TAT ($r = -0.43, P = 0.106$), and a significant negative correlation of this capacity with $F_{1+2}$ ($r = -0.62, P = 0.013$, Figure 6D).

**Figure 7.** Inflammatory response and relation to granulocyte microparticles (GMP). Median and range of the data in the healthy individuals ($n = 14$) are presented, together with the individual data of patients 1 - 9. Plasma concentrations of A. IL-6, B. CRP, C. Elastase and D. Correlation between the number of GMP and plasma concentration of elastase.
Inflammatory response and relation to circulating microparticles

To assess the extent of the inflammatory response in the patients, plasma concentrations of the cytokine IL-6, the acute phase reactant CRP and the granulocyte product elastase were determined. Figure 7 (A-C) shows the individual concentrations in each patient and median plus range values in the healthy controls. The plasma concentrations of IL-6 and CRP were clearly elevated in all patients as expected. Elastase concentrations were also above the 97.5 percentile of the control range in 6 out of 9 patients. The number of GMP found in patient plasma samples correlated with their plasma elastase concentrations ($r = 0.70, P = 0.036$; Figure 7D), but not with the concentrations of CRP ($r = 0.05, P = 0.898$) or IL-6 ($r = -0.14, P = 0.787$).

DISCUSSION

The present study shows that patients with MODS and sepsis have relatively low numbers of circulating microparticles. Reconstitution of these microparticles in normal plasma evoked less thrombin generation than microparticles from controls, and the mechanisms of thrombin formation clearly differed from healthy individuals.

Coagulation in vivo is generally believed to be initiated by the complex of TF and factor VIIa [141], which results in the conversion of prothrombin (factor II) -via factor X(a)- into active thrombin (factor IIa). There is extensive evidence supporting the relevance of this coagulation activation pathway in vivo: (i) the bleeding tendency in persons with hereditary deficiencies, (ii) infusion of activated factor VII into chimpanzees increases the concentrations of the activation peptides of factors IX, X and II, which increases are blocked by infusion of anti-TF [118], and (iii) infusion of anti-TF prevents the development of DIC in baboons that receive lethal doses of E. coli [119]. In our previous studies we also found that thrombin generation induced by microparticles, isolated from either material of the pericardial cavity of patients undergoing cardiopulmonary bypass [33] or from the systemic circulation of a patient with meningococcal septic shock and DIC [29], occurred via a TF/factor VII(a)-dependent mechanism. In the present study, however, anti-TF partially inhibited thrombin generation in only four patients. In two patients, thrombin generation was strongly inhibited by anti-factor XII, which in our previous studies was completely
ineffective [29,33], despite the fact that this antibody completely blocks kaolin-induced generation of thrombin in normal plasma [139]. The role of factor XII in coagulation is still obscure. Patients with hereditary factor XII deficiency do not have a bleeding tendency [113,114] and administration of anti-factor XII does not prevent the development of DIC in baboons after administration of lethal doses of E. coli [118]. Our present findings suggest that microparticles can activate factor XII. Whether this factor XII-activating property is explained by differences in composition or cellular origin of the microparticles remains to be determined. We can only speculate on the cellular origin of the microparticles involved in activation of factor XII. Factor XII is present on the neutrophil membrane surface [118,142,143]. Interestingly, the two patients that showed by far the strongest inhibition of thrombin generation by anti-factor XII (patients 1 and 5) also had the highest numbers of GMP and the highest plasma elastase concentrations of the nine patients studied. A relation between the GMP and the factor XII activation in an in vitro thrombin generation assay could therefore be hypothesized.

In eight of the nine patients, anti-factor XI MoAb strongly inhibited thrombin generation, more than observed with the healthy controls. The role of factor XI in coagulation, as with factor XII, has long been debated. Patients with hereditary factor XI deficiencies suffer in various extents from bleeding abnormalities [144]. Factor XI can be activated by factor XIIa and by thrombin [75]. Once activated, factor XIa induces more extensive thrombin formation -via factors IXa and Xa- and then also indirectly inhibits fibrinolysis in plasma [116]. Factor XI can be activated on the platelet surface in the absence of factor XII [145]. Despite the fact that in patients the numbers of PMP were decreased, they still comprise about 40% of the total number of circulating microparticles and possibly provide an efficient surface for activation of factor XI. Platelets contain a unique form of factor XI, platelet factor XI, which is an alternative splicing product of the plasma factor XI gene [134] and which is present on the platelet surface [135]. To our knowledge, it is unknown whether platelet factor XI and plasma factor XI differ in their sensitivity for activation via either factor XIIa or thrombin. It is equally unknown whether microparticles differentially cause activation of plasma and platelet factor XI.
Development of MODS may be due to hypercoagulation and platelet deposition, leading to obstruction of blood vessels and finally organ dysfunction [146]. The platelet count in the patients was significantly lower than in the controls (patients 121 x 10^9/L [median], range 54 - 223; controls 238 x 10^9/L, range 172 - 276; P < 0.01), whereas the numbers of PMP per platelet were comparable (P = 0.477). This could suggest that the number of PMP in the circulation of patients and healthy individuals are simply a reflection of the number of platelets. Alternatively, the low numbers of PMP in the patients may be due to deposition of platelets- and/or PMP in the organs [87,147]. Deposition of PMP has been observed in the microvasculature after porous balloon delivery [148] and in atherosclerotic plaques [108].

The numbers of PMP found in the controls in the present study (237 x 10^6/L) are much higher than those reported previously by us (41 x 10^6/L) [29]. In the earlier study, we used EDTA-anticoagulated plasma that had been frozen and stored for 5 - 9 years at -70 °C but that had not been snap frozen prior to storage, whereas in the present study citrate anticoagulated plasma was used that had been snap frozen in liquid nitrogen and that was used within 1 - 2 months. This implies that absolute numbers of cell-derived microparticles are likely to be dependent on conditions of collection and storage. However, especially the snap freezing of the plasma aliquots was noted to be important to reliably quantify microparticles. It emphasizes that appropriate samples from healthy controls should always be included in clinical studies, and conditions of collection, preparation and storage carefully noted in manuscripts.

In the present study the number of EMP in the healthy controls using the anti-E-selectin antibody (64 x 10^6/L) is also much higher than in our previous report (18 x 10^6/L). The detection of EMP is cumbersome due to the lack of appropriate antibodies. Combes et al. used antibodies directed against PECAM-1 and α3β3, i.e. non-endothelial cell specific antibodies [56]. We used an antibody directed against E-selectin (CD62E), a protein only expressed on activated endothelial cells. This antibody was titrated on microparticles that strongly expose E-selectin, i.e. microparticles isolated from IL-1α stimulated human umbilical vein endothelial cells, to ensure a proper antibody concentration. Compared to IgG control antibody staining,
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microparticles bound this anti-E-selectin antibody although their fluorescence intensity was just above the fluorescence threshold of the IgG control. Although these microparticles may expose E-selectin, we question their endothelial cell origin. Very recently, we obtained plasma samples from 12 patients with systemic lupus erythematosus, and found subpopulations of microparticles strongly binding anti-E-selectin in 3 patients. Staining with an antibody directed against VE-cadherin (CD144), like E-selectin a protein expressed only by endothelial cells, also showed a strong staining of a microparticle subpopulation in only these three patients, thus confirming their endothelial cell origin (data not shown). The anti-CD144 data therefore seem more reliable than the anti-E-selectin data to estimate the number of EMP.

The numbers of annexin V-positive EMP and those exposing TF were also reduced compared to controls, possibly via similar mechanisms as stated above. GMP (CD66b) were absent in plasma samples in healthy controls, but clearly present in the patients. By far the highest number of GMP did not stain for annexin V. The presence of these solely marker-positive events seems specific, since they are entirely absent in control plasma samples. At present, one can only speculate about the function and genesis of these distinct subpopulations. Only the CD66b- and annexin V-positive population correlated with elevated levels of elastase ($r = 0.70, P = 0.036$), and not the annexin V-negative population ($r = 0.41, P = 0.273$), but we presume both populations to originate from granulocytes. Previously, we reported circulating GMP in patients with meningococcal septic shock [29] and the present study demonstrates that GMP are not restricted to those patients. The plasma concentrations of IL-6, CRP and elastase were elevated in (almost) all patients, but only elastase correlated to the number of GMP, strengthening the observation that circulating GMP indeed reflect activation of granulocytes in the circulation.

The microparticle fractions from patients, containing microparticles of various cellular origins, generated on average less thrombin in normal plasma than microparticles from controls. Patients, however, clearly had enhanced coagulation activation in vivo, as reflected by increased concentrations of $F_{1-2}$ and TAT, and an inverse correlation was present between the numbers of microparticles and plasma
Microparticles from patients with MODS and sepsis

TAT concentrations. This apparent discrepancy between thrombin generation in vitro and enhanced coagulation in vivo may be explained in two ways. First, the more extensive inhibition of the microparticle-induced thrombin generation by anti-factor XI may indicate that the microparticles in the patients, although present in low numbers, are in some way more capable of supporting the XI loop of coagulation activation [149]. Similarly, the extensive inhibition by anti-factor XII and anti-TF in 2 and 4 patients, respectively, points to different coagulation activation properties of the microparticles in the patients versus healthy individuals. Second, (increased) adherence of microparticles to endothelial cells, monocytes or other circulating blood cells, resulting in a reduction of their circulating numbers, may initiate expression and production of TF and cytokines. In vitro, leukocyte microparticles stimulate endothelial cells to produce TF and IL-6 [60], whereas PMP can stimulate monocytes [150]. As a consequence, the production of, for instance, TF by target cells would result in enhanced coagulation activation in vivo and its reported TF dependence [118,119,141].

Taken together, the present study shows that in patients with MODS and sepsis the circulating numbers of microparticles are relatively low, and differ in cellular origin from those in healthy controls. These patient microparticles initiate thrombin generation via different mechanisms from those described previously for microparticles in pericardial blood of patients undergoing cardiopulmonary bypass and patients with meningococcal sepsis.

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