Circulating Endothelial Cells as a Prognostic Marker in Thrombotic Microangiopathy

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Background: Circulating endothelial cells (CECs) are a reliable marker of disease activity in a variety of vascular disorders. Damage to microvascular endothelial cells is a hallmark of thrombotic microangiopathy (TMA). The aim of this study is to identify and count CECs during the course of TMA and evaluate whether cell numbers may serve as a prognostic marker in patients undergoing plasma exchange.

Methods: Fifteen patients (8 women, 7 men) aged 31 to 66 years with TMA of different causes were studied before and after 4 sessions of plasma exchange. CECs were isolated by using anti-CD146–driven immunomagnetic isolation and counted after staining with Ulex Europaeus lectin-1.

Results: Numbers of CECs were markedly elevated in all patients before treatment (64 to 672 CEC/mL; mean, 320 ± 205 CEC/mL) compared with healthy controls (0 to 16 CEC/mL; mean, 6.4 ± 4.2 CEC/mL; \(P < 0.001\)). Patients with a favorable outcome had significantly greater initial CEC levels (mean, 426 ± 175 CEC/mL; \(P < 0.001\)), and cell numbers decreased significantly after 4 treatments of plasma exchange (mean, 101 ± 53 CEC/mL; \(P = 0.001\)). Patients with disease unresponsive to plasma exchange presented with lower initial CEC levels (mean, 108 ± 36 CEC/mL), and numbers failed to decrease after plasma exchange (mean, 114 ± 57 CEC/mL; \(P = 0.827\)).

Conclusion: Markedly elevated numbers of CECs reflect severe and widespread endothelial damage in patients with TMA. Cell numbers at presentation and their degree of decrease after 4 sessions of plasma exchange could provide important prognostic clues.

INDEX WORDS: Circulating endothelial cells; thrombotic microangiopathy; prognostic marker; endothelial damage.
Histopathologic findings of TMA are strikingly similar regardless of the underlying cause, and severe endothelial damage is the hallmark of TMA: Subendothelial widening of the capillary wall, endothelial swelling and necrosis, and microvascular thrombi are characteristic features. In analogy to vasculitis, we hypothesized that damaged endothelial cells undergo detachment from the basement membrane and become detectable in peripheral blood. Anecdotal evidence from a single case report prompted us to speculate that cell numbers reflect the severity of endothelial damage in TMA. Finally, plasma exchange is the cornerstone of treatment in patients with TMA and response to treatment is highly variable; therefore, we were interested to correlate cell numbers at presentation and during the course of treatment with clinical response to plasma exchange.

**Methods**

**Patients**

This study was conducted in accordance with the Declaration of Helsinki, and the local institutional review board approved the study protocol. Informed consent was obtained before blood collections. We studied 15 patients (8 women, 7 men; age, 31 to 66 years; mean, 47 years) with a diagnosis of TMA. The diagnosis of TMA was established by the presence of microangiopathic hemolytic anemia with fragmented red blood cells, thrombocytopenia, and sufficient evidence of end-organ damage, such as renal failure or neurological abnormalities. Each patient underwent an extensive workup to exclude other causes for these findings. Blood samples from 15 healthy volunteers (age, 25 to 63 years; mean, 48 years) were obtained as controls.

**Plasmapheresis**

Plasma separators were used to perform plasmapheresis. Exchange volumes were approximately 35 to 40 mL/kg body weight. Volume ranged from 2.5 to a maximum of 4 L per treatment. Fresh frozen plasma was used as exchange volume. The number of treatments depended on the patient’s clinical response.

**Blood Samples**

A total of 61 blood samples were collected from the 15 patients at various times during their disease course. Peripheral blood was obtained before the first plasma exchange and before the next 4 treatments, when possible. One to 4 serial samples were obtained. Samples were stored at 4°C when necessary, but analyzed within 4 hours of venipuncture. Care was taken to perform nontraumatic venipuncture and discard the first tube of blood, as discussed elsewhere.

Another blood sample was obtained before the first plasma exchange and for analysis of vWFcP (Miha Furlan, Central Hematology Laboratory, University Hospital, Inselspital, Bern, Switzerland). In addition, we obtained blood for an assay of factor H antigen.

**Isolation and Enumeration of CECs**

CECs were isolated and counted as described elsewhere. Briefly, CECs were isolated from whole blood by using Pan-Mouse M450 Dynabeads (Dynal, Oslo, Norway) coated with anti-CD146 antibody (Biocytex, Marseille, France), as described previously. Nonspecific binding of beads to leukocytes was prevented with rigorous washing and FcR blocking agent (Miltenyi, Bergisch Gladbach, Germany). Cells subsequently were incubated with Ulex Europaeus lectin-1 (UEA-1). We used either rhodamine-coupled (Linaris, Wertheim, Germany) or fluorescein isothiocyanate–coupled (Sigma, Deisenhofen, Germany) UEA-1 (2 mg/mL) for 1 hour under dark conditions. Samples were washed in the magnet, and cells were suspended in buffer. Cells were counted under fluorescence microscopy by using a Nageotte counting chamber. UEA-1–positive particles larger than 10 μm with more than 4 beads attached were regarded as CECs. Conglomerates were counted as 1 cell. Presence of a nucleus was not a criterion for counting because anuclear endothelial carcasses were noted previously.

To study cell morphological characteristics, we stained cells with acridine instead of UEA-1 and visualized cells by using fluorescence microscopy. To further confirm the endothelial origin of cells, we stained with anti-CD31 antibodies instead of UEA-1. For this purpose, we used an alkaline phosphatase–antialkaline phosphatase kit (Dako Diagnostika, Hamburg; Germany) and sheep antihuman CD31 antibodies (The Binding Site, Heidelberg, Germany) with fluorescein isothiocyanate donkey antiseep secondary antibody (The Binding Site).

**Statistical Analysis**

Student t-test was used to detect significant differences between patients and healthy controls. Differences between CEC numbers before plasma exchange between patients with favorable and unfavorable outcomes also were evaluated by using paired Student t-test. Repeated-measurement analysis (Wilk Λ) was used to determine whether there was a significant decrease in CECs over time in regard to outcome. The same test was applied looking at number of thrombocytes. Data are presented as mean ± SD.

**Results**

**Characterization of Patients**

Five patients (mean age, 43 years; 2 men, 3 women) had TMA associated with bone marrow transplantation. One patient (a man aged 37 years) had diarrhea-associated TMA with a stool assay positive for enterohemorrhagic *E. coli*. Three other patients (mean age, 55 years; 2 women) had TMA associated with a malignancy. Two of
those patients received mitomycin chemotherapy, which also may cause TMA. Four of 14 patients had decreased vWFcP levels (mean age, 49 years; 3 men, 1 woman); 2 of these patients had circulating inhibitor. After comprehensive workup, the cause of TMA remained unclear in 2 patients (31 and 61 years old, 2 women; Table 1). A factor H assay was performed in 14 of 15 patients, and none of those patients had a deficiency.

Ten of 15 patients improved clinically after treatment with plasma exchange, which we interpreted as a favorable outcome. Three patients died during the first 2 days of their illness despite initiation of treatment with plasma exchange. Two patients did not improve after several plasma exchange treatments. The outcome of these 5 patients is defined as unfavorable (Table 1). Twelve of 15 patients had renal involvement. Four of those patients remained on hemodialysis therapy, and 2 patients developed chronic renal insufficiency. Six patients had recovery of renal function (Table 1).

### Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Cause of TMA</th>
<th>Outcome</th>
<th>vWFcP</th>
<th>Creatinine at Discharge (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>34</td>
<td>BMT (B-NHL), TMA: 5 mo after aPBSCT</td>
<td>Clinically improved</td>
<td>Normal</td>
<td>0.83*</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>34</td>
<td>BMT (ALL), TMA: 18 d after aPBSCT</td>
<td>Clinically improved</td>
<td>Normal</td>
<td>0.97*</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>38</td>
<td>Decreased vWFcP activity</td>
<td>Clinically improved</td>
<td>25% w/o CI</td>
<td>0.81*</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>62</td>
<td>Decreased vWFcP activity &amp; circulating inhibitor</td>
<td>Clinically improved</td>
<td>5% with CI</td>
<td>0.84*</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>46</td>
<td>Decreased vWFcP activity &amp; circulating inhibitor</td>
<td>Clinically improved</td>
<td>3% with CI</td>
<td>0.70*</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>51</td>
<td>Decreased vWFcP activity</td>
<td>Clinically improved</td>
<td>25% w/o CI</td>
<td>0.95*</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>37</td>
<td>EHEC</td>
<td>Clinically improved</td>
<td>Normal</td>
<td>HD</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>55</td>
<td>Malignancy (gastric cancer), mitomycin/5-FU</td>
<td>Improved</td>
<td>NA</td>
<td>3.96†</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>31</td>
<td>Unknown</td>
<td>Improved</td>
<td>Normal</td>
<td>HD</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>61</td>
<td>Unknown</td>
<td>Improved</td>
<td>Normal</td>
<td>2.99‡</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>40</td>
<td>BMT (ALL), TMA: 10 wk after aPBSCT</td>
<td>Unchanged</td>
<td>Normal</td>
<td>HD</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>46</td>
<td>BMT (CML), TMA: 9 mo after aPBSCT</td>
<td>Unchanged</td>
<td>Normal</td>
<td>HD</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>62</td>
<td>BMT (MDS, secondary AML), TMA: 18 d after aPBSCT</td>
<td>Died</td>
<td>Normal</td>
<td>HD</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>44</td>
<td>Malignancy (anal cancer), mitomycin/5-FU &amp; decreased vWFcP activity</td>
<td>Died</td>
<td>3% with CI</td>
<td>HD</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>66</td>
<td>Malignancy (mediastinal mass of unknown cause), no chemotherapy administered</td>
<td>Died</td>
<td>Normal</td>
<td>HD</td>
</tr>
</tbody>
</table>

NOTE. To convert creatinine in mg/dL to μmol/L, multiply by 88.4.

Abbreviations: BMT, bone marrow transplant; CI, circulating inhibitor; EHEC, enterohemorrhagic E coli; PE, plasma exchange; HD, hemodialysis required; NA, not available; CRI, chronic renal insufficiency; w/o, without; B-NHL, B-cell non-Hodgkin lymphoma; ALL, acute lymphocytic leukemia; CML, chronic myelogeneous leukemia; MDS, myelodysplastic syndrome; aPBSCT, allogenic stem cell transplantation.

*Stable creatinine level at 3 months to 4 years.
†Died 4 months later (no autopsy, TMA unlikely).
‡Creatinine level 4.5 years later, 2.32 mg/dL.

### Enumeration of CECs

Using immunomagnetic isolation, we identified a population of UEA-1–positive cells, as described previously (Fig 1). Cells were 10 to 100 μm in size and had variable morphological characteristics. The majority of cells were round or oval when studied with acridine orange alone. CD31 staining further confirmed the endothelial origin of these cells. As described previously, staining of isolated CECs with CD3, CD8, CD14, and CD45 was negative. This virtually excludes T lymphocytes and leukocytes as the origin of the circulating cells enumerated with the CD146-driven immunomagnetic isolation.

Anuclear carcasses, conglomerates of several cells, also were seen. The phenotype of the cells suggests that the vast majority of cells were not viable, although formal testing for apoptosis/necrosis was not carried out.

CEC numbers were markedly elevated in all patients before treatment (320 ± 205 CEC/mL; range, 64 to 672 CEC/mL) compared with healthy
controls (6.4 ± 4.2 CEC/mL; range, 0 to 16 CEC/mL; *P* < 0.001; Fig 2A).

Ten patients who improved clinically had significantly greater CEC levels at presentation (426 ± 175 CEC/mL; *P* < 0.001) compared with patients with an unfavorable outcome (108 ± 36 CEC/mL; *P* < 0.001; Fig 2B).

Eleven patients had 5 serial samples. In patients treated successfully, CEC levels decreased significantly after 4 treatments of plasma exchange from a mean of 428 ± 187 CEC/mL (range, 164 to 672 CEC/mL) to 101 ± 53 CEC/mL (range, 36 to 137 CEC/mL; *P* = 0.001; Fig 2C). CEC numbers in patients with an unfavorable outcome did not decrease (*P* = 0.827). Thrombocytes increased significantly after 4 treatments of plasma exchange in patients who improved clinically (*P* = 0.047; Table 2). Patients with an unfavorable outcome did not have a significant decrease in platelet count (*P* = 0.806). Lactate dehydrogenase levels decreased in patients with a favorable outcome (*P* < 0.03; Table 2).

Repeated-measurement analysis (significance measurement per Wilk $\lambda$) was used to assess the decrease in CEC numbers over time, thus looking at all serial samples. *P* for the comparison of patients with favorable and unfavorable outcomes is 0.05 (*P* per Wilks $\lambda$). No significant changes in platelet counts (*P* > 0.2) or lactate dehydrogenase levels (*P* > 0.3) were observed.

### DISCUSSION

Microvascular endothelial damage is the histopathologic hallmark of TMA. Light and electron microscopy show widespread endothelial edema and necrosis. Endothelial damage is induced by various factors, and a genetic basis for some forms of TMA has been established. Activation of neutrophils and/or platelets is crucial to the pathogenesis, and verotoxin-1, a cause of diarrhea-associated TMA, promotes leukocyte adhesion to cultured endothelial cells. Antienothelial antibodies also were implicated. Markers of endothelial damage in peripheral blood have not been studied in great detail, although endothelial microparticles were described in patients with thrombotic thrombocytopenic purpura. To date, CECs in patients with TMA were described in only a single case report.

In the current study, we describe CECs as a novel marker of endothelial damage in patients with TMA. Cell numbers were markedly elevated in all patients with TMA compared with healthy controls. Cell numbers were in the same range as in patients with active untreated small-vessel vasculitis, indicating a similar degree of widespread endothelial damage. The highly variable cell phenotype with anuclear carcasses, smaller particles, and fragments is further evidence of the severity of the disease process. Finally, it must be noted that elevated numbers of
CECs were a universal finding in all our patients with TMA regardless of the underlying cause.

Untreated TMA in adults usually is progressive and commonly results in renal failure or death from multiorgan failure. Accordingly, mortality before the introduction of plasma exchange was as high as 90%. Even with this treatment option, TMA carries a mortality rate of 22% to 31%. Twenty-six percent of our patients died despite treatment. Nevertheless, plasma exchange remains the cornerstone of treatment in patients with TMA. One possible explanation for the effectiveness of this treatment modality is the correction of protease deficiencies in patients with TMA caused by vWFcP disturbance. However, other forms of TMA also respond. Accordingly, removal of pathogenic autoantibodies and other unknown circulating factors, such as proinflammatory cytokines, has been postulated.

In this study, successful plasma exchange, indicated by improvement in clinical manifestation and thrombocytopenia, was reflected by a decrease in CEC numbers. The half-life of CECs presently is unknown. It is conceivable that the rapid decrease in cell numbers during successful plasma exchange reflects a relatively short life span of CECs in peripheral blood. In this regard, we previously showed a rapid decrease in CEC

Fig 2. CEC numbers in (A) 15 healthy controls and 15 patients with TMA at their initial presentation, (B) 10 patients with favorable outcome and 5 patients with unfavorable outcome shown as box plots, and (C) the 2 patient groups (favorable versus unfavorable outcome; error bars [at 5 different times; 1, initial presentation; 2-5, days 2 to 5 before plasma exchange]). Abbreviation: CI, 95% confidence interval.
numbers after steroid treatment for acute vascular rejection in renal transplant recipients. Alternatively, CECs may be removed by the plasma exchange procedure itself. Interestingly, initial cell counts in the subgroup of patients that ultimately responded favorably to plasma exchange were higher compared with those who did not respond. Taken together, our findings suggest that cell numbers before treatment with plasma exchange could serve as a prognostic marker. We speculate that lower CEC levels in patients with TMA that does not respond to plasma exchange reflect a different pathophysiology. We observed relatively low cell numbers at presentation in 3 of 5 patients who developed TMA caused by hematopoietic stem-cell transplantation. The lack of decrease in CEC numbers in the group of patients with a poor prognosis who underwent plasma exchange suggests that the mechanism of decrease is not caused by removal by plasma exchange, but rather decreased “shedding” from the vasculature. In addition, there was no significant decrease in CEC numbers during the treatment course in these patients. Our findings confirm that TMA caused by hematopoietic stem-cell transplantation is a unique and unpredictable disorder that often is refractory to treatment. The other 2 patients with unfavorable outcome were diagnosed with a malignancy and received chemotherapy. Studies of similar patients confirmed that patients with malignancy-associated TMA usually have a poor prognosis. Conversely, 4 of 5 patients with decreased vWFcP responded well to plasma exchange. These patients had high numbers of CECs at presentation that decreased significantly after plasma exchange. These findings are supported by clinical experience that patients with TMA caused by severe vWFcP deficiency respond well to plasma exchange.

In summary, we establish CECs as a marker of endothelial damage in patients with TMA. Our findings might provide a novel tool to guide the diagnostic workup, assess the prognosis, and evaluate treatment options. Our results warrant additional studies to confirm the utility of this novel marker in a clinical setting with a larger number of patients.

ACKNOWLEDGMENT

The authors thank Heide Regelsberger, Hanne Gros, and Baerbel Maess for excellent technical help and Ludwig Hoy for support regarding the statistical analysis.

REFERENCES


Table 2. Absolute CEC and Platelet Counts and Lactate Dehydrogenase Levels Before and After Serial Plasmapheresis

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Initial CEC (cells/mL)</th>
<th>Initial Platelets (Tsd/μL)</th>
<th>Initial Lactate Dehydrogenase (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>582</td>
<td>296</td>
<td>210</td>
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<tr>
<td>2</td>
<td>672</td>
<td>129</td>
<td>87</td>
</tr>
<tr>
<td>3</td>
<td>477</td>
<td>291</td>
<td>108</td>
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<tr>
<td>4</td>
<td>168</td>
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<td>5</td>
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<td>561</td>
<td>762</td>
<td>1,060</td>
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<td>416</td>
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</tr>
<tr>
<td>15</td>
<td>99</td>
<td>111</td>
<td>Died</td>
</tr>
</tbody>
</table>

NOTE. Absolute counts of CECs and platelets and lactate dehydrogenase levels before and after serial plasmapheresis. C1 to C4, P1 to P4, and L1 to L4: times of 4 consecutive treatments of plasmapheresis. Abbreviation: NA, not available.


