Circulating Inflammatory Endothelial Cells Contribute to Endothelial Progenitor Cell Dysfunction in Patients with Vasculitis and Kidney Involvement

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Impaired angiogenic function has been reported in patients with kidney failure. During vascular damage, endothelial cells may detach from the site of inflammation and be released into the peripheral blood. With the use of Wegener’s granulomatosis as a study model, whether circulating inflammatory endothelial cells (IEC) can (1) be used as a disease activity marker and (2) contribute to sustained vascular damage by inducing endothelial progenitor cell (EPC) dysfunction were examined. IEC—defined as endothelial cells that express the two inflammatory-associated markers vascular-adhesion protein-1 (VAP-1) and MHC class I-related chain A (MICA)—were increased significantly in patients with active disease as compared with those in remission. IEC expressed high levels of inducible nitric oxide synthase and neutrophil-activating chemokines, such as macrophage inflammatory protein-1α, growth-related oncogene-α, epithelial neutrophil activating peptide-78, and IL-8, and induced increased neutrophil migration. IEC levels significantly correlated with C-reactive protein and extent of organ involvement. Patients with active disease had decreased numbers of EPC colony-forming units and a high expression of VAP-1 and MICA in kidney endothelium. EPC did not express VAP-1 or MICA. IEC significantly inhibited proliferation, migration, and endothelial nitric oxide synthase expression in EPC. Thus, apart from being a new disease activity marker, IEC may contribute to vascular damage by impairing the functional capacity for repair by EPC. IEC may provide a unique in vitro system to study pathogenesis of kidney and vascular diseases.


Circulating endothelial cells (CEC) have been described in various conditions with vascular injury, including chronic renal failure and systemic vasculitis (1–10). These studies indicate the existence of two types of cells in peripheral blood: endothelial progenitor cells (EPC) that are bone marrow derived and inflammatory endothelial cells (IEC) that are thought to be detached from the vessel walls and enter the circulation as a result of vascular injury. So far, EPC in peripheral blood have been identified using mAb against various specific surface markers, including vascular endothelial growth factor receptor-2 (VEGFR-2) (11–13). Markers for IEC have not been identified so far.

Wegener’s granulomatosis (WG) is one form of systemic vasculitis of unknown cause (14). The disease is life-threatening and involves necrotizing granulomatous inflammation of the upper and/or lower respiratory tract and necrotizing glomerulonephritis. It is well documented that the endothelium is the primary target of injury in WG (15,16). The presence of antineutrophil cytoplasmic antibodies (ANCA) found in 95% of these patients may have an important role in the damage of endothelial cells in vasculitis (17). In addition, antibodies directed to antigenic components situated on the surface of endothelial cells have been implicated in WG (18).

Clinicopathologic and experimental findings indicate that the vascular inflammation is the primary process in vasculitis (14–16). This suggests that disease activity and the extent to which IEC are released from lesions of ongoing/recent inflammation might result in increased numbers of circulating endothelial cells, which can be used as disease/injury activity marker. We therefore hypothesized that the number of IEC released from these lesions might reflect the extent and the severity of organ involvement. An additional goal of this study was to gain insight into the possible contribution of circulating IEC in the progression of WG disease. We therefore tested the potential functional capacity of circulating IEC isolated from patients with active WG and compared it with cells that were isolated from patients in clinical remission. To distinguish IEC from EPC, we tested the expression of two novel markers of endothelial cell inflammation, vascular-adhesion protein-1 (VAP-1) and MHC class I-related chain A (MICA). MICA is a stress...
antigen upregulated mainly on epithelial cells as well as certain endothelial cells, keratinocytes, fibroblasts, and monocytes (19). VAP-1 is an endothelial adhesion molecule that possesses semi-carbazide-sensitive amine oxidase activity that is translocated to endothelial cell surfaces upon inflammation, where it mediates leukocyte interactions with endothelium (20). Unlike MICA, VAP-1 is not expressed on leukocytes, monocytes, epithelial cells, or fibroblasts. However, the biologic function of this molecule remains unknown. Both of these molecules are expressed in the kidney during inflammation (21,22). Thus, in our report, WG was used as a study model to test the hypothesis that circulating IEC (1) could be phenotypically distinguished from endothelial progenitor cells, (2) be used as a disease/injury activity marker and, (3) contribute to sustained vascular damage by inducing endothelial progenitor cell dysfunction.

Materials and Methods

Patients
We studied a total of 36 patients who had WG and were positive for proteinase 3 (PR3)-ANCA. Disease entity was defined using the Chapel Hill consensus on the nomenclature of systemic vasculitides (23), and disease activity was defined according to the Birmingham vasculitis activity score (24). Some demographic and clinical data are presented in Table 1. The patients were divided into two groups. Group 1 consisted of 16 patients with newly diagnosed active disease. Blood samples were collected before initiation of immunosuppressive treatment. Group 2 consisted of 20 patients in clinical remission. Twenty healthy, normal control subjects (mean age 49; range 30 to 60 yr; 10 men and 10 women) were included in the study. Patients were recruited upon examination at their hospital unit, and informed consent from all individuals was obtained at blood sampling. C-reactive protein (CRP) was measured by nephelometry. Hemoglobin, leucocyte count, serum creatinine, and proteinuria were measured at blood sampling. C-reactive protein (CRP) was measured by nephelometry. Hemoglobin, leucocyte count, serum creatinine, and proteinuria were measured at blood sampling. C-reactive protein (CRP) was measured by nephelometry.

Flow Cytometric Analysis of VAP-1+ and MICA+ Cells
Endothelial cell sampling and analysis were performed on the same day. Peripheral blood mononuclear cells (PBMC; 5 × 10⁷) that were isolated from patients with WG and healthy individuals were labeled with antibodies against either VAP-1 (S. Jalkanen) or MICA (Amgen, Seattle, WA) as described earlier (25). PE-conjugated goat-anti mouse IgG antibodies (Jackson IRL, Baltimore, MD) were used as secondary antibody. The secondary antibody was also used as negative control. We also double-stained the cells for VAP-1 (antibody isotype IgG2a) and MICA (isotype IgG1) using isotype specific secondary antibodies goat anti-mouse IgG2a (PE conjugated) and anti-mouse IgG1 (FITC conjugated; Jackson Immunoresearch, Baltimore, MD). FITC-annexin V/propidium iodide kit (BD Pharmingen, San Diego, CA) was used to identify apoptotic and necrotic cells. Cells were analyzed on a FACSorter (Becton Dickinson, Franklin Lakes, NJ). Fluorescence signals from 10,000 cells were counted, and the percentage of positively stained cells in gated events was recorded.

A two-step colony-forming assay was performed to isolate and detect the number of EPC as described by Hill et al. (26). Confirmation of endothelial cell lineage was performed by indirect immunostaining using endothelial-specific antibodies directed against VEGFR-2 and endothelial-nitric oxide synthase (eNOS; BD Pharmingen). Furthermore, after 1 wk of cultivation, the EPC were stained by immunocytochemistry as described earlier (25), for CD133, MICA, VAP-1, and inducible nitric oxide synthase (iNOS; BD Transduction Lab, Franklin Lakes, NJ).

Isolation and Characterization of VAP-1+/MICA+ Cells
Our flow cytometric analysis indicated that in all instances, >97% of VAP-1+ cells also expressed MICA. We therefore isolated VAP-1+ cells to characterize the nature of these cells. We isolated these cells from 6 × 10⁶ PBMC of patients with WG (n = 5 in active disease and n = 5 in remission) and five healthy individuals using antibodies to VAP-1. The VAP-1 antibodies first were coupled to magnetic particles (Miltenyi Biotec, Gladbach, Germany). The procedure was performed as described by the manufacturers. Necrotic cells were removed by the dead cell removal kit using the procedure described by the manufacturers. The VAP-1–coated magnetic particles were added to the PBMC populations depleted of necrotic cells, and the VAP-1+ cells were isolated according to the procedure described by the manufacturers. To confirm expression of activated endothelial and inflammatory markers on VAP-1+ and MICA+ cells, we stained the cells with antibodies to −CD45 (FITC), −CD14 (PE), −CD2 (FITC), CD68 (PE), CD106 (FITC), CD62E (PE), CD142, and CD144 (BD Pharmingen); CD105 (FITC), CD141, von Willebrand factor (vWF; FITC), CD31 (FITC), and Ulex europaeus (FITC; Sigma-Aldrich, Munich, Germany); and acetylated-LDL (Ac-LDL; FITC; Molecular Probes, Inc., Eugene, OR) and CD133 (Miltenyi Biotec). Control antibodies included mouse anti-human fibroblast and anti-α–actin antibodies from Harlan Sera-Lab (Loughborough, UK) and mouse anti-human epithelial antigen (epithelial cell adhesion molecule) antibodies from Dakopatts AB (Alvsjö, Sweden). The cells were analyzed using the flow cytometer as described earlier. Immunocytochemical staining (25) of VAP-1+/MICA+ cells with antibodies against VAP-1, MICA, vWF, CD133, and iNOS was also performed. VAP-1+/MICA+ cells were tested for viability, adherence to fibronectin-coated (20 μg/ml) tissue culture plates, and proliferation in endothelial selective medium MCDB 131 (Life Technologies, Gaithersburg, MD) that contained 5% heat-inactivated human AB serum, 5 mM t-glutamine, and 100 μg/ml penicillin/streptomycin. The medium was supplemented further with endothelial cell growth medium singlequots (Clonetics, BioWhittaker, Walkersville, MD; MCDB complete medium).

In Vitro Angiogenesis Assay
Formation of capillary tubelike structures by VAP-1+/MICA+ cells was assessed in matrigel, which frequently is used for the evaluation of in vitro angiogenesis. The assay was performed as described earlier (11). Human umbilical vein endothelial cells were used as positive control.

Functional Analysis of VAP-1+/MICA+ Cells
VAP-1+/MICA+ cells (5 × 10⁵) were cultured in serum-free endothelial growth medium (Life Technologies, Stockholm, Sweden) and 5 × 10⁵ in MCDB complete medium. Supernatants from all cultures were collected at 72 h and stored at −70°C for further analysis. The following experiments were performed to elucidate some functions for the VAP-1+/MICA+ cells in WG.

Detection of Chemokines. Because a role for neutrophils has been implicated in the pathogenesis of WG, we tested serum-free supernatants of VAP-1+/MICA+ cell cultures from patients with WG and healthy control subjects for the presence of chemokines that may activate and recruit neutrophils. We therefore measured levels of macrophage inflammatory protein-1α (MIP-1α), IL-8, growth-related oncogene-α (GRO-α), and epithelial neutrophil activating peptide-78 (ENA-78), using commercially available ELISA kits (R&D Systems,
The assay was performed as described by the manufacturers.

**Neutrophil Migration Assay.** We further tested whether the supernatants from the VAP-1/H11001/MICA/H11001 cell cultures from patients with WG and healthy control subjects had the ability to induce neutrophil migration using a 48-well Boyden chamber (Neuro Probe, Gaithersburg, MD). Neutrophils were isolated from 10 normal individuals, and the assay was performed as described earlier (27,28). Migrated cells were counted in four randomly chosen microscope fields (×40) in a blinded manner, and the average was determined.

**Proliferation and Migration Assays for EPC.** Finally, supernatants from VAP-1/H11001/MICA/H11001 cultures were also tested for their effect on proliferation, eNOS expression, and migration of EPC. EPC colonies were obtained from 50 ml blood of healthy subjects (n = 10) as described earlier, and day 3 colonies were treated for 72 h with VAP-1+/MICA+ culture supernatants from patients who had active disease or were in remission. We prepared single-cell suspensions of the colonies using trypsin-EDTA, and the cells were divided equally into three fractions. One cell fraction was treated with the 5-bromo-2-deoxyuridine (BrdU; 30 mM; Sigma-Aldrich) for 30 min. Percentage of proliferating cells was determined using a FACScan (BD Biosciences, San Jose, CA) and the CellQuest software (BD Biosciences). Migration of EPC was measured using a modified Boyden chamber assay as described earlier (29). The assay was performed in triplicate, and the data were expressed as the mean ± SEM.
ating BrdU\textsuperscript{7} cells was determined using a FITC-conjugated anti-BrdU antibody (Sigma-Aldrich) and analyzed by flow cytometry. The second cell fraction was stained for eNOS by indirect immunolabeling using an eNOS-specific antibody (BD Transduction Lab).

The rest of the cells were used in a Boyden chamber migration assay as described above. Cells were tested for migration toward 50 ng/ml concentration of VEGF (R&D Systems). Supernatant from EPC colonies that were not treated with VAP-1+/MICA+ cell culture supernatants were used as controls in all assays.

**Immunohistochemical Staining for VAP-1 and MICA in Kidney Sections**

Frozen kidney sections (5 μm) from two patients with active and confirmed kidney involvement were stained with anti–VAP-1 or anti–MICA according to standard immunohistochemical procedure as described earlier (11). The diaminobenzidine tetrahydrochloride DAB-Nickel substrate kit was used as color developer. Sections were counterstained with hematoxylin and mounted in mounting media (ImmunKemi, Stockholm, Sweden). Normal kidney biopsy specimens were used for comparison. Immunofluorescence staining using FITC-conjugated (green) and Cyanine 3-conjugated (red) antibodies was performed as described earlier (11).

**Statistical Analyses**

Data are presented as mean ± SD or median and range as appropriate, with $P < 0.05$ indicating statistical significance. For comparison among three groups for nonnormal distributed variables, we used Kruskal Wallis test, and when ANOVA was significant, we performed Dunnett’s test for multiple comparisons between the groups. For nominal variables, comparisons between groups were made by $\chi^2$ test. Correlations were performed by the Spearman rank test (Rho). All data were analyzed with SAS version 9.1 for Windows statistical software (SAS, Cary, NC).

**Results**

**Increased Numbers of Circulating VAP-1+/MICA+ Cells in Patients with WG during Active Disease**

Patients with active WG displayed significantly higher numbers of VAP-1+/MICA+ cells as compared with patients who were in remission ($P < 0.01$) and healthy individuals ($P < 0.001$; Figure 1, A and B). In terms of the number of VAP-1+/MICA+ cells/ml of blood, Figure 1A data correspond to approximately 50, 100, and 180 cells/ml in control subjects, patients with WG in remission, and patients with active WG, respectively. We found by double staining that the majority (>95%) of VAP-1+ cells also expressed MICA (Figure 1B). The cells co-stained with anti-CD144 and -CD106 but not -CD3 or -CD45 antibodies (Figure 1C). We also found that patients with active disease have a high percentage of necrotic cells (40.0 ± 6.2 propidium iodide positive) as compared with those in remission (20.0 ± 5.6) and healthy control subjects (8.0% ± 3.4).

![Figure 1](image-url)
EPC Do Not Express VAP-1 or MICA

We found that the numbers of EPC colony-forming units were significantly lower in patients with active WG as compared with those in remission \( (P = 0.006; \text{Figure 2A}) \) and healthy individuals \( (P = 0.007) \). The EPC colonies were positive for VEGFR-2 and eNOS, confirming the endothelial phenotype of the cells (Figure 2B). In addition, the EPC formed tubules in matrigel, indicating endothelial functional capacity (Figure 2B). Importantly, these cells did not stain for VAP-1, MICA, or iNOS but stained positively for the endothelial progenitor cell marker CD133 (Figure 2B).

VAP-1+/MICA+ Cells Are Circulating Endothelial Cells

The viability of the isolated VAP-1+/MICA+ cells was >95%. These cells adhered to fibronectin-coated plates within 3 to 5 h and formed a monolayer within 3 to 4 d. The cells, however, did not exhibit any extensive proliferative capacity, and after 10 d in culture, the cells became senescent and detached from the plastic surface. Furthermore, the numbers of isolated VAP+ cells/ml of blood (approximately 175 cells/ml) was consistent with the flow cytometry results. Phenotypic characterization of VAP-1+/MICA+ cells demonstrated that these cells expressed all of the markers of mature activated endothelial cells (Table 2) but did not express CD14, CD68, CD45, or the EPC marker CD133. Immunocytochemical analysis confirmed expression of VAP-1 and MICA on these cells from patients with active disease (Figure 3A). These cells expressed high levels of iNOS as compared with those in remission \( (n = 10; \text{Figure 3A}) \). Furthermore, these cells formed tubules in matrigel, indicating the endothelial origin of these cells (Figure 3B).

Functional Effects of Supernatants from Circulating VAP-1+/MICA+ Cells

We found higher levels of IL-8, ENA-1, MIP-1α, and GRO-α (Table 3) in supernatants from patients with active disease as compared with those in remission \( (P < 0.05) \) and healthy control subjects \( (P < 0.05) \). Similarly, we found higher numbers of
chemotactic neutrophils that migrated toward VAP-1+/MICA+ cell culture supernatants from patients with active disease as compared with those in remission ($P < 0.008$; Figure 3, C and D). In comparison with VAP-1+/MICA+ cell supernatants from patients in remission, supernatants from patients with active disease had an inhibitory effect on the expression of eNOS (Figure 4A) and proliferation ($P < 0.01$; Figure 4B) of EPC that were isolated from normal, healthy individuals. Furthermore, these VAP-1+/MICA+ cell culture supernatants inhibited the ability of healthy EPC to migrate in response to the angiogenic growth factor VEGF ($P < 0.01$; Figure 4C). These results indicate that VAP-1+/MICA+ cells are IEC.

**In Vivo Expression of VAP-1 and MICA on Kidney Endothelium**

One of us has reported previously (19) that VAP-1 is expressed in peritubular capillaries in normal kidneys. Here, we observed no expression of MICA in normal kidneys. However, we found that MICA was strongly expressed in vessels and in epithelial cells, whereas VAP-1 was strongly expressed in the peritubular and tubular capillaries and around vessel areas but weakly in kidney glomeruli of patients with active disease. Kidney endothelium stained positively for both markers. We

**Table 2. Phenotypic characterization of circulating VAP-1+/MICA+ cells isolated from patients with active WG**

<table>
<thead>
<tr>
<th>Antibodies to</th>
<th>VAP-1+/MICA+ % Cells Expressing Marker ($n = 5$)</th>
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<tbody>
<tr>
<td>MICA</td>
<td>+ (97 ± 2)</td>
</tr>
<tr>
<td>CD45/CD14</td>
<td>/ / - (0)</td>
</tr>
<tr>
<td>CD68</td>
<td>- (0)</td>
</tr>
<tr>
<td>CD133</td>
<td>- (0)</td>
</tr>
<tr>
<td>CD142</td>
<td>+ (53 ± 6)</td>
</tr>
<tr>
<td>CD144</td>
<td>+ (95 ± 2)</td>
</tr>
<tr>
<td>Acetylated LDL</td>
<td>+ (100 ± 0)</td>
</tr>
<tr>
<td><em>Ulex europaeus</em></td>
<td>+ (100 ± 0)</td>
</tr>
<tr>
<td>CD106</td>
<td>+ (75 ± 5)</td>
</tr>
<tr>
<td>CD62E</td>
<td>+ (56 ± 7)</td>
</tr>
<tr>
<td>CD31</td>
<td>+ (100 ± 0)</td>
</tr>
<tr>
<td>vWF</td>
<td>+ (97 ± 2)</td>
</tr>
<tr>
<td>CD105</td>
<td>+ (100 ± 0)</td>
</tr>
<tr>
<td>CD141</td>
<td>+ (97 ± 3)</td>
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<tr>
<td>Epithelial cell marker</td>
<td>EPCAM - (0.05 ± 0.01)</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>- (0)</td>
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<tr>
<td>α-Actin</td>
<td>- (0)</td>
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$^a$VAP-1, vascular-adhesion protein-1; MICA, MHC class I-related chain A; vWF, von Willebrand factor.

Figure 3. Characterization of circulating IEC in patients with WG. (A) Circulating IEC (VAP-1+/MICA+) isolated from patients with active WG (AWG) express the markers VAP-1 (red), MICA (black), VAP-1 and MICA (red/black; double-stained), iNOS (black), the endothelial marker von Willebrand factor (vWF; red), but not CD133 or VEGFR-2. (B) Formed tubules in matrigel, confirming the endothelial phenotype of these cells. Human umbilical vein endothelial cells are used as control. (C) Supernatants of circulating IEC cultures from patients with AWG induced significantly increased migration of neutrophils as compared with RWG culture supernatants. (D) A representative picture of migrated neutrophils in a Boyden chamber assay from a patient with AWG and an RWG patient. Note the increased number of migrated cells (pink clusters) in response to supernatants from IEC of AWG as compared with RWG. Magnification, ×40 in A; ×10 in B.
Table 3. Chemokine levels (pg/ml) in supernatants of circulating IEC obtained from healthy control subjects and patients with WG at debut and in remission

<table>
<thead>
<tr>
<th></th>
<th>Healthy control subjects</th>
<th>Patients with WG at debut</th>
<th>Patients with WG in remission</th>
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<tbody>
<tr>
<td></td>
<td>IL-8</td>
<td>ENA-78</td>
<td>MIP-1α</td>
</tr>
<tr>
<td>1</td>
<td>&lt;30</td>
<td>&lt;30</td>
<td>&lt;30</td>
</tr>
<tr>
<td>2</td>
<td>&lt;30</td>
<td>&lt;30</td>
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<tr>
<td>3</td>
<td>&lt;30</td>
<td>&lt;30</td>
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<tr>
<td>4</td>
<td>100 ± 31</td>
<td>&lt;30</td>
<td>&lt;30</td>
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<tr>
<td>5</td>
<td>435 ± 13</td>
<td>175 ± 11</td>
<td>56 ± 12</td>
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<td>&lt;30</td>
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aData are expressed as mean ± SD. IEC, inflammatory endothelial cells; MIP-1α, macrophage inflammatory protein-1α; GRO-α, growth-related oncogene-α; ENA-78, epithelial neutrophil activating peptide-78.

Further confirmed the expression of MICA and VAP-1 on kidney endothelium by double staining with an endothelial cell marker CD31 (Figure 5, A through J).

Correlations of Circulating Endothelial Cells with Inflammatory and Renal Function Markers

Finally, we performed Spearman rank analysis to determine whether the frequency of VAP-1+/MICA+ cells was associated with some laboratory markers of inflammation and renal function, namely hemoglobin levels, leukocyte count, S-creatinine, and CRP. We found that the numbers of VAP-1+/MICA+ cells were inversely correlated with hemoglobin levels ($p = -0.51, P = 0.001$) and positively with leukocyte count ($p = 0.5, P = 0.015$) and CRP ($p = 0.41, P = 0.01$; Figure 6A). Furthermore, VAP-1+/MICA+ cells were significantly and positively correlated with the number of organs involved ($p = 0.53, P = 0.001$; Figure 6B). No correlation between the number of IEC or other laboratory parameters with Birmingham vasculitis activity score was obtained.

Discussion

Evidence suggests that in WG, endothelial cells undergo necrosis and detach from the site of inflammation and are released into the peripheral blood (9). Mechanisms by which vascular damage is repaired are not well understood; however, it is well documented that EPC may play an important role (29,30). No previous attempts have been made to isolate and study whether detached IEC play any role in the sustained inflammatory response.

We initiated this study by testing the expression of two endothelial inflammatory molecules, VAP-1 and MICA, to distinguish IEC from EPC. IEC did not express the endothelial progenitor cell marker CD133; however, staining with constitutive mature and activated endothelial cell markers showed consistent expression of vWF, VE-cadherin, tissue factor, VCAM, E-selectin, etc. The cells lacked expression of the leucocyte marker CD45 and monocyte marker CD14. Thus, VAP-1 and MICA may be used as reliable markers of inflammation on endothelial cells. In addition, we found that kidney endothelium in vivo strongly expressed VAP-1 and MICA during active disease, indicating that the circulating IEC may indeed be cells detached from sites of injury. VAP-1 is found in inflamed lung (31), and we have observed expression in nasal endothelium (unpublished observations). Thus, the three major organs that are known to be affected in WG, namely nose, lungs, and kidneys, express VAP-1.

We report that during active WG, the frequency of IEC is significantly increased, whereas treatment results in a significant decrease. These observations led us to believe that circulating IEC may be cells detached from sites of injury and thus reflect functional capacity of the cells at these sites. We further postulated that these cells might participate in maintaining the aberrant immune activation observed in WG and may simultaneously have an inhibitory effect on EPC. Our subsequent results showed that IEC expressed high levels of iNOS and produced chemokines that are known to recruit and activate neutrophils. In fact, increased expression of iNOS and decreased expression of eNOS in renal endothelium of patients with WG has been reported (32). Severe endothelial damage with invasion of neutrophils and necrosis is a salient feature of WG (17). Interaction between activated endothelial cells and primed neutrophils seems to be a pathogenetic factor of impor-
tance for the vascular lesions. Thus, endothelial cells in affected organs may play an important role in mediating inflammation by increased iNOS (33) production and recruiting and activating neutrophils via chemokine production. In addition, because a large number of these cells are necrotic, they may be responsible for raised systemic concentrations of various endothelial proteins, such as vWF and thrombomodulin observed in WG (16).

In our study, IEC produced soluble factors, which had a significant negative effect on the proliferation, migration, and eNOS expression of EPC. In addition, we found a markedly decreased yield of EPC colonies from peripheral blood samples of patients with active WG as compared with those who were in remission. Thus, these cells may have impaired functional capacity. The exact nature of the soluble factors is currently unknown. Whether VAP-1 via its enzymatic function may be responsible for these effects is currently being investigated.

Our study demonstrated that the numbers of IEC per milliliter of blood were consistent with the flow cytometry results indicating that the use of flow cytometry for detection of IEC is a reliable method and can replace the more tedious method of mechanical isolation and enumeration of these cells from blood. We found a higher number of IEC (average 180 cells/ml) in patients with active WG as compared with mean absolute values of CEC (140 cells/ml blood) in a similar patient group as reported by Woywodt et al. (8,9). Numbers of CEC >50/ml in control subjects and >180/ml in patients have been described in other studies (2,6–8). This discrepancy may be due to the use of different methods and markers used for identification of endothelial cells (10). Woywodt et al. used antibodies to CD146 (P1H12) to isolate and enumerate circulating endothelial progenitor cells. However, in our experience, the antibody P1H12 (cat. no. 550314; BD Pharmingen) was not found to be specific for CEC but also stained circulating activated T cells from patients with active WG (unpublished observations).

So far, studies in this area have been hindered by the inaccessibility of vascular endothelium in patients. Our results suggest that circulating IEC are useful material for the study of endothelial cells in the pathogenesis of WG. We recently reported that a significantly high fraction of these patients have
anti-endothelial cell antibodies specific for kidney, lung, and nasal endothelium (18). In a preliminary study, we found that autoantibodies from these patients also show intense staining (black) for VAP-1 in peritubular and tubular endothelium. For expression of VAP-1 in normal kidney specimens, see ref. 21. (E and F) Immunofluorescence staining shows that both VAP-1 (FITC, green) and MICA (FITC, green) double stain with an endothelial cell marker CD31 (Cy3, red). (G) A glomerulus double stained for an epithelial cell marker (epithelial cell adhesion molecule [EPCAM], green) and MICA (red). (H) Enzymatic staining of the same section also shows positive staining for VAP-1 (brown). (I) Double-staining of kidney endothelium for VAP-1 (red) and MICA (green). (J) Control staining with only secondary FITC and Cy3-labeled antibodies. In E through J, DAPI was used to stain the nucleus blue. Magnification, ×40 in A through F and I; ×60 in G, H and J.

Figure 5. Detection of VAP-1 and MICA in biopsies of patients with active WG. (A and B) Immunohistochemical staining with control and MICA antibodies show no positive staining in a normal kidney biopsy. (C) Positive staining (black) for MICA in peritubular endothelium and glomerular epithelial cells of the kidney in patients with WG during active disease. (D) Kidney specimens from these patients also show intense staining (black) for VAP-1 in peritubular and tubular endothelium. For expression of VAP-1 in normal kidney specimens, see ref. 21. (E and F) Immunofluorescence staining shows that both VAP-1 (FITC, green) and MICA (FITC, green) double stain with an endothelial cell marker CD31 (Cy3, red). (G) A glomerulus double stained for an epithelial cell marker (epithelial cell adhesion molecule [EPCAM], green) and MICA (red). (H) Enzymatic staining of the same section also shows positive staining for VAP-1 (brown). (I) Double-staining of kidney endothelium for VAP-1 (red) and MICA (green). (J) Control staining with only secondary FITC and Cy3-labeled antibodies. In E through J, DAPI was used to stain the nucleus blue. Magnification, ×40 in A through F and I; ×60 in G, H and J.

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for repair. On the basis of our results, we suggest that circulating IEC may contribute to the pathogenesis/progression of WG by interfering with the functional capacity for vessel wall repair by endothelial progenitor cells. Furthermore, we demonstrate that VAP-1 and MICA are novel markers of endothelial inflammation that may help distinguish inflammatory from progenitor endothelial cells.

Acknowledgments
This study was financed by grants from the Medical Research Council, grants 00793 to Prof. Möller and K2002-06X-14004-02B to S.S.-H.

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Figure 6. Clinical correlations of circulating IEC in patients with WG. (A) In a Spearman rank analysis, the numbers of circulating IEC (VAP-1+/MICA+ cells) inversely correlated with hemoglobin levels and positively with C-reactive protein. (B) Furthermore, IEC were significantly and positively correlated with the number of organs involved ($\rho = 0.53, P = 0.001$) in patients with WG.


