UNINFECTED AND CYTOMEGALIC ENDOTHELIAL CELLS IN BLOOD DURING CYTOMEGALOVIRUS INFECTION: EFFECT OF ACUTE REJECTION

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Abstract
After transplantation cytomegalovirus infections can cause vascular damage in both the graft and the host. To study a possible relationship between the degree of vascular injury, clinical symptoms of HCMV infection and transplant rejection, we investigated the appearance and numbers of endothelial cells (EC) in blood of 54 kidney transplant recipients in a prospective clinical study. We identified two types of endothelial cells: cytomegalic endothelial cells (CEC) which were detected in patients with moderate or high HCMV antigenemia, uninfected EC were observed in patients during HCMV infection as well as without HCMV infection. The incidence of either CEC, EC or the combination of both was associated with HCMV-related clinical symptoms (P<0.01). Remarkably, the occurrence of rejection episodes before HCMV infection was an important risk factor for the occurrence of endothelial cells in blood (EC, CEC, or both) during HCMV infection (P<0.001).

Introduction
Human cytomegalovirus (HCMV) infection is one of the most common infectious complications in kidney allograft recipients and may cause severe morbidity [1]. In vivo as well as in vitro observations have shown HCMV infected endothelial cells which could be involved in viral dissemination [2,3]. Infected endothelial cells can occasionally detach from the basal membrane, enter the bloodstream and can be detected in the peripheral blood of HCMV patients [4,5]. These cytomegalic cells have a diameter of 35-45 µm and contain nuclear inclusion bodies. Clumps of cytomegalic endothelial cells were demonstrated [6]. The permissively infected cells may have a role in viral dissemination or can be involved in organ damage [5]. The incidence of CEC varies between different immunosuppressed populations [5,7]. CEC in peripheral blood have been found to be associated with high viral load and organ involvement [5] although this could not be confirmed by others [4,7]. In this prospective study of patients with HCMV infection after renal transplantation we studied the relationship between the appearance of distinct endothelial cells in blood, HCMV disease symptoms and transplantation rejection episodes. Isolated mononuclear cell fractions on cytopsots were studied by immunocytological staining for the presence of endothelial cells. Further investigation with markers for different stages of HCMV infection were used to examine whether aside CEC, endothelial cells in earlier stages of infection could also detach and gain access to the peripheral blood.

Patients and methods
Consecutive patients after renal transplantation were prospectively studied for HCMV infection as defined by HCMV antigenemia. Patients with positive HCMV antigenemia for less than 1 –2 weeks were excluded (n=12) as well as patients with vascular damage not related to HCMV infection (n=3). Rejection episodes were diagnosed according to the Banff criteria [8]. Treatment consisted of methylprednisolone, followed by a course of ATG in case of steroid resistant rejection (Merieux, Lyon, France). Vascular rejection episodes were treated with ATG and plasmapheresis. Patients were monitored twice a week for HCMV
antigenemia. The HCMV antigenemia test was performed according the procedure recently reviewed for standardization [1].

No HCMV- prophylaxis such as ganciclovir, acyclovir or hyperimmune gammaglobulin was given. Fourteen patients received ganciclovir because of clinical symptoms associated with rising HCMV antigenemia values.

Blood samples to study the occurrence of endothelial cells were obtained before HCMV infection at approximately 15 days after transplantation and weekly after the first positive HCMV antigenemia test result. This was continued until the HCMV antigenemia was negative (n=32) or less than 5/50,000 cells (n=12). Blood samples of patients without HCMV infection (n=10) were studied at approximately 15, 40, 50 and 60 days post transplantation.

CEC in PB were analyzed according to a quantitative method as described previously [4,9]. Briefly, heparinized blood samples were obtained by venapuncture. The mononuclear cell (MNC) fraction was isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma AS, Oslo, Norway). 1 x 10^5 MNC were cytocentrifuged per slide. For each sample, a variable number of cytospots was analyzed depending on the concentration of MNC/ml blood. Four cytospots were analyzed if the concentration of MNC/ml blood was 1.5 x 10^6 or less, otherwise 6 to 8 cytospots were analyzed. The number of analyzed slides represented a detection limit of 20 CEC/ml blood in 95% of all samples. This standardization of blood volume was chosen to circumvent effects of leukopenia or leukocytosis. In a previous report we showed a recovery of 45% of EC from blood [9]. This correction factor was included in the calculation. The following monoclonal antibodies were used for staining of the cytospots: C10/C11 directed against HCMV pp65 and E1/1 2.3 directed to a 90kD cell surface antigen of endothelial cells [10]. EC were stained with E13 directed against HCMV IE proteins (Seralab, Sussex, UK). Fixation was with 1% paraformaldehyde, followed by indirect immunofluorescence doublestaining with fluorescein isothiocyanate (FITC) or tetramethyl rhodamine (TRITC) (SBA, Birmingham, USA) for endothelial specific markers and HCMV antigens, respectively. CEC and EC were determined by counting double positive cells or FITC-positive cells only.

Statistical analyses were performed with contingency tables (χ²-test), non-parametric Mann-Whitney test or non-parametric analysis of variance (Kruskal Wallis) for differences in distribution between groups, differences between two groups and differences between multiple groups, respectively.

Results

Fifty-four patients were included in this study (32 male, 22 female, median age 45 years, range 18 - 71). In total 320 samples were analyzed (median samples per patient 5, range 2 - 15). Patients were stratified into four groups depending on the highest obtained HCMV antigenemia: none, low (1-10 pp65+PMNs/50000), moderate (11-100) or high (>100). Patients of group 4 (13/16) and group 3 (5/12) with high viral loads had clinical symptoms such as fever, malaise, leukocytopenia, thrombocytopenia and elevated liver enzymes.
<table>
<thead>
<tr>
<th>HCMV-pp65 Antigenemia*</th>
<th>N</th>
<th>group</th>
<th>primary</th>
<th>Secondary</th>
<th>symptoms</th>
<th>vascular</th>
<th>steroid resistant</th>
<th>steroid sensitive</th>
</tr>
</thead>
<tbody>
<tr>
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<td>10</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Low: 1-10</td>
<td>16</td>
<td>2</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Moderate: 11-100</td>
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<td>3</td>
<td>3</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>High: &gt;100</td>
<td>16</td>
<td>4</td>
<td>10</td>
<td>6</td>
<td>13</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>54</td>
<td></td>
<td>13</td>
<td>31</td>
<td>18</td>
<td>5</td>
<td>10</td>
<td>13</td>
</tr>
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</table>

*No. positive granulocytes/50.000
Figure 1
Frequencies (A, B) and concentrations (C, D) of CEC (A, C) and EC (B, D) in peripheral blood of HCMV patients. The frequency is the number of patients per group with (C)EC at any moment during infection. Hatched parts of a bar represent patients with HCMV infections only and the black parts of a bar represent patients with both preceding acute rejection episodes and HCMV infection.

Twenty-eight patients had one or more rejection episodes: 13 patients experienced interstitial rejection responding to steroid treatment, 10 patients had steroid resistant interstitial rejection and 5 patients had vascular type of rejection. Episodes of vascular rejection were associated with high viral load: 0/5 in group 1 or group 2 versus 5/5 of group 3 and 4. Patients with one or more rejection episodes were equally distributed among all groups (P=0.41)(Table 1).

Two distinct types of endothelial cells in peripheral blood were observed: late stage infected cytomegalic endothelial cells (CEC) and uninfected endothelial cells (EC). We never observed endothelial cells in immediate early or early stages of HCMV infection. Both CEC and EC were detectable in blood at or just after the maximum HCMV antigenemia peak. After maximum HCMV antigenemia, EC could be detected for a longer time than CEC. In 3 of 10 patients without HCMV infection, EC were demonstrated. Two of these patients had
EC at 15 days post transplantation, which was shortly after a rejection episode. The other patient experienced neither rejection nor HCMV infection. Remarkably, in the patients with HCMV infection, all EC were detected during HCMV antigenemia and never before HCMV infection.

CEC were detected in 11/44 patients with HCMV infection (Fig. 1A) (25.0%): 8/16 patients in group 4 (50.0%) and 3/12 patients in group 3 (25.0%) (Fig. 1A). Concentrations of CEC ranged from 0.11 / ml to 30.26 / ml (median 0.89 / ml) (Fig. 1C). In 4 patients of group 4 CEC were detected at various times during HCMV antigenemia (data not shown). EC were observed in all patient categories independent of the severity of infection (Fig. 1B). The concentrations of EC ranged from 0.17 EC / ml blood to 114.05 EC / ml blood (median 2.62 EC / ml blood)(Fig. 1D).

Patients with rejection episodes had endothelial cells in blood (EC, CEC, or both) during HCMV infection more often (66.7%), than patients without rejection (15.0%)(P<0.001). The detection of endothelial cells was not significantly related to the type of rejection. A tendency could be observed to higher (C)EC frequencies in patients with a more severe type of rejection (6/13 of patients with interstitial rejection versus 12/15 of patients with vascular rejection or steroid resistant interstitial rejection).

Patients with CEC had significantly more HCMV-associated clinical symptoms (81.8%) than patients without CEC (27.3%) (P<0.01). Eleven out of 16 HCMV patients with EC had HCMV-associated symptoms (68.8%) as compared to 7 of the remaining 28 patients (25.0%) (P<0.01). Fourteen patients from groups 3 and 4 were treated with ganciclovir, of which 7 patients had detectable CEC. Ten out of 14 patients had clinical symptoms and were treated with ganciclovir.

**Discussion**

This study demonstrates that the appearance of CEC as well as EC is related to HCMV antigenemia levels as well as HCMV associated symptoms. Intriguingly, patients with acute rejection episodes and HCMV infection had higher considerably higher frequencies of endothelial cells in peripheral blood.

In our study we detected CEC only in patients with moderate or high viral load, which was comparable to the study of Percivalle et al [5]. In that study the CEC numbers of individual patients were higher. This may have been due to the greater immunosuppression given to these heart-lung transplant recipients, resulting in higher viral loads and, consequently, higher numbers of CEC. In contrast, bone marrow transplant patients may already have CEC at low levels of HCMV antigenemia, with comparable numbers of CEC as seen in the present study [7]. Obviously, factors such as the type of transplantation, immunosuppression, or whether pre-emptive HCMV treatment was given influenced not only the course of HCMV infection, but also endothelial involvement.

Release of uninfected EC has been described for several abnormalities with vascular injury such as sickle cell anemia [11]. These authors describe endothelial cells in circulation in healthy individuals [11]. With the procedure used in our study we were not able to detect endothelial cells in the blood of healthy individuals (data not shown).
The occurrence of EC in peripheral blood was closely related to active HCMV infection, even though these cells are not infected. It is unknown why these cells are released. Recently, animal models demonstrated endothelial progenitor cells originating from the bone marrow in peripheral blood. These cells were capable of homing to vascular lesions [12]. Characteristically, these cells were positive for CD34, but also for CD45. In our study the EC observed during HCMV infection were negative for CD45, making it unlikely that they were bone marrow derived.

Detection of CEC, EC or both in HCMV patients was strongly related to the occurrence of earlier rejection episodes. CEC were mainly observed in patients with high HCMV antigenemia. In addition to a specific inflammatory reaction in the graft, acute rejection is followed by a generalized inflammatory response. Plasma levels of different cytokines are elevated, including TNFα. Binding of TNFα could stimulate the HCMV immediate early promoter/enhancer region and thus enhance the infectivity of that cell by HCMV [13].

It is also possible that the EC originate from pre-existing endothelial lesions in the transplanted graft, probably enhanced by HCMV. Especially during vascular rejection, damage is directed at the endothelium. In our study 4 out of 5 patients with vascular rejection had endothelial cells during HCMV infection. According to the Banff criteria [8] only arterial involvement is a criterion for interstitial rejection (Banff criteria for kidney transplants). However, the occurrence of venous involvement (venulitis) could also contribute to detectable endothelial damage [14]. In our center we have observed that biopsy-proven interstitial rejection of kidney transplants with evident venulitis frequently requires ATG treatment (manuscript in preparation) and represented a more severe form of interstitial rejection. Furthermore, because of the sampling error in taking biopsies, vascular lesions at different sites in the graft could be missed.

In conclusion, the occurrence of CEC, EC or both in peripheral blood is related to HCMV antigenemia and HCMV-associated clinical symptoms. Transplant rejection mechanisms and HCMV infection have a cumulative effect on the release of endothelial cells. Many studies have shown that both HCMV infection and acute rejection are risk factors for chronic transplant failure [15]. With these data we demonstrate that multiple injury in the first weeks after transplantation have cumulative effects at the endothelial cell surface, which may predispose these patients towards chronic graft failure.

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References


