Vascular pathophysiology of cytomegalovirus infection after kidney transplantation

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ACUTE REJECTION BEFORE CMV INFECTION ENHANCES VON WILLEBRAND FACTOR AND sVCAM-1 IN BLOOD

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Submitted
Abstract

Human cytomegalovirus (HCMV) infections in transplantation patients are associated with vascular endothelial damage. This is reflected by the appearance of cytomegalic endothelial cells (CEC) and non-infected endothelial cells in blood. To get more insight in the extent of vascular damage during HCMV infection we investigated levels of soluble markers during HCMV infection in relation to endothelial cell levels but also the preceding acute rejection episodes.

Of 46 kidney transplant patients, plasma levels of Von Willebrand Factor (vWF), sICAM-1, sVCAM-1 and sE-sel were analyzed during the course of HCMV infection. Plasma levels of vWF and sVCAM-1 were two-fold increased during severe HCMV infection. Moreover, the plasma levels of vWF correlated to detectable cytomegalic and non-infected endothelial cells in blood. The kinetics of changes in vWF and endothelial cells (CEC and EC) demonstrated the relationship with HCMV induced vascular damage. Levels of sICAM-1 and sE-sel in plasma did not significantly change during HCMV infection. Interestingly, the combination of HCMV infection and preceding acute transplant rejection caused the highest increases of vWF and sVCAM-1 plasma levels, reflecting an enhanced susceptibility for endothelial damage at the moment of infection. Furthermore, these patients may have an increased risk for chronic transplant dysfunction.

Introduction

HCMV infections are an important cause of morbidity in the post transplant period [1] and may result in a HCMV associated syndrome. Infected vascular endothelial cells may contribute to HCMV related organ dysfunction and dissemination of the virus. Aside from acute effects of HMCV infection, chronic infection of endothelial cells may underlie intragraft as well as systemic vascular pathology in the long run. Hence, the possible role of HCMV infection in chronic transplant dysfunction or accelerated arteriosclerosis attracted recent interest [2,3].

Acute infection of endothelial cells is directly evidenced by the appearance of cytomegalic endothelial cells (CEC) in peripheral blood of patients with active HCMV infection [4-6]. The occurrence of CEC was related to the severity of infection and organ involvement [5], although the two other studies could not confirm this finding [4,6]. In a previous study of kidney transplant recipients with HCMV infection, we found that CEC only occurred in patients with moderate to high HCMV antigenemia and correlated with HCMV associated clinical symptoms [7]. Furthermore, in these patients also non-infected EC (EC) were observed during HCMV infection. This indicates that not only infection itself caused endothelial damage, but also a more generalized form of endothelial damage was induced. Until now, no data were available whether occurrence of CEC and/or EC in blood is reflected in plasma parameters of endothelial activation or endothelial damage.

Apart of cytomegalovirus infections many factors may cause endothelial damage to both the allograft and the recipient, such as reperfusion injury, acute rejection episodes or thrombotic complications. As a result of chronic transplant dysfunction the allograft may be lost.
Damage as well as inflammatory stimuli induce the release of von Willebrand Factor (vWF) by endothelial cells, and the serum level of vWF is considered to be one of the best markers available for endothelial damage [8-12]. Intercellular adhesion molecule-1 (ICAM-1), an adhesion molecule expressed on many cell types, is upregulated upon endothelial activation by cytokines such as IL-1 and TNFα. This activation also leads to increased shedding of the soluble form, sICAM-1 [13]. Vascular cell adhesion molecule (VCAM-1) is induced after activation by cytokines. It is thought to be more endothelium specific than sICAM-1, although dendritic cells, macrophages, vascular smooth muscle cells and epithelia express VCAM-1 as well. Finally, E-selectin (E-sel) is exclusively found on activated endothelium. Both increased sICAM-1 and sVCAM-1 are associated with allograft rejection and HCMV disease [14,15]. Levels of sE-sel did not correlate with acute rejection episodes [16,17] nor with HCMV infection [17]. However, the data concerning HCMV infection are based on a few number of patients. Considering the specificity of E-sel for activated endothelium, we included measurement of sE-sel in our study.

In the present study we focussed on acute rejection episodes and active HCMV infection in a prospective design. We hypothesized that the appearance of CEC and EC in patients with symptomatic HCMV infection would be related to increased levels of plasma parameters vWF, sICAM-1, sVCAM-1 and sE-sel. For this, we analyzed and correlated the increases in plasma levels of the soluble parameters with HCMV viral load, CEC, EC and clinical symptoms. Acute rejection not only results in increased plasma levels of vWF, sVCAM-1 and sICAM-1 [12,13,15], but during HCMV infection it causes a higher incidence of endothelial cells in blood and thus endothelial damage [7]. Hence, we also studied the relationship of changes in plasma levels during HCMV infection and preceding acute rejection episodes that occurred before HCMV infection.

Patients and methods

Patients

Sixty-five consecutive patients were eligible for our prospective study after renal transplantation (35 male, 30 female, median age 48 years (range 18 – 70 years). Exclusion criteria were HCMV antigenemia at the start of the prospective study (15 days post transplantation) (n=4) or a short period of HCMV antigenemia (no samples obtained) (n=12); endothelial damage due to trombotic thrombocytopenic purpura or hemolytic uremic syndrome (n=1) and requirement of percutaneous transluminal coronary angioplasty (n=2). Forty-three patients received a cadaveric transplant; three patients received a kidney from a living related donor. Initial immunosuppression consisted of cyclosporin A (Novartis, Basle, Switzerland) and low dose prednisolone; twenty patients additionally received mycophenolate mofetil (Roche, Basle, Switzerland). Seven patients received an induction course of OKT3 (Jansen Cilag, Belgium) or anti-thymocyte immunoglobulin (ATG) (Fresenius, Oberursel, Germany), either because of high anti-HLA antibodies or retransplantation. Rejection was diagnosed according Banff criteria [18]. Interstitial rejection was treated with 1 gram methylprednisolone intravenously on three consecutive days (Solu-Medrol: Upjohn, Kalamazoo, MI), followed by 5 courses 4 mg/kg of ATG given on
alternative days (Merieux, Lyon, France) in case of steroid resistant rejection. Vascular rejection was treated with ATG and plasmapheresis. Thirty patients, who had IgG antibodies against HCMV late antigen before transplantation, were considered seropositive for HCMV [19]. The HCMV antigenemia was routinely assessed twice a week starting at day 15 after transplantation. No HCMV- prophylaxis like ganciclovir, acyclovir or hyperimmune globulin was given. Ten patients who developed a moderate to severe HCMV infection received ganciclovir at early clinical symptoms and/or rising HCMV antigenemia values. Plasma samples were collected at day 15 after transplantation and weekly after onset of HCMV antigenemia until it became negative (n=29) or less than 5 pp65 positive PMNs / 50,000 cells (n=7), approximately 60 – 100 days after transplantation. Plasma samples of patients without HCMV infection were sampled at day 15, day 40, day 50, and day 60.

**HCMV antigenemia**

The HCMV antigenemia test was performed according the procedure recently reviewed for standardization [1]. Briefly, peripheral blood leukocytes were dextran-sedimented followed by lysis of erythrocytes with NH₄Cl. After two washes the leukocytes were counted and cytopsots were prepared. Cytopsots were fixed with paraformaldehyde, followed by a permeabilization step with NP40. Indirect peroxidase staining was with C10/C11, a mixture of two mouse monoclonal antibodies directed to HCMV pp65 [20]. Pp65 positive cells per spot were counted, whilst the number of negative cells per spot was determined by automated image analysis. The HCMV antigenemia score was calculated from the number of positive cells per 50,000 leukocytes. Two spots were analysed for each patient sample. In earlier work we showed that the antigenemia score correlated with viral load [21]. Patients were divided into four groups, based on the maximum obtained HCMV antigenemia values by the individual patients. These were no, low (1-10 pp65^+PMN/50000 cells), moderate (11-100) and high (>100), respectively groups 1 - 4.

**Von Willebrand Factor**

Von Willebrand Factor (vWF) levels were measured in citrated plasma by an enzyme-linked immunosorbent assay (ELISA) using commercially available antibodies (Dakopatts, Glostrup, Denmark). In short, microtiter plates (Immunoplate Maxisorb, Nunc, Roskilde Denmark) were coated with polyclonal rabbit-anti-human vWF. The plates were incubated with plasma samples diluted 1:50 and 1:200 or standard control plasma (dilution range 1.25 % to 500% of a 1:100 prediluted plasma), followed by a HRP-conjugated polyclonal rabbit anti human vWF antibody. The last step was a colorimetric reaction with ortho-phenylene-diamine (OPD), after which OD could be measured. Results are expressed as percentages of a standard composed of pooled human plasma.
Soluble ICAM-1 ELISA
Soluble ICAM-1 (sICAM-1) in EDTA-plasma was measured using a commercially available sICAM-1 module kit (Bender MedSystems, Vienna, Austria). In brief, ELISA plates (Nunc Immunoplate Maxisorb, Roskilde Denmark) were incubated with a monoclonal antibody directed against ICAM-1. Unbound sites were blocked with 0.5% BSA, 0.05% Tween. Plasma samples at a dilution of 1:100 or serial twofold dilution of sICAM-1 standard protein (ranging from 0.63 ng/ml to 10.0 ng/ml) were applied. HRP-conjugated anti-ICAM-1 monoclonal antibody was used as detection antibody and tetramethyl benzidine (TMB) as substrate for the enzyme reaction. According the standard the sICAM-1 concentrations in the samples could be calculated. If the sample OD exceeded the OD of the standard, the sample concentration was adjusted to 1000 ng/ml. Five samples obtained during HCMV infection of five patients were set at 1000 ng/ml.

Soluble VCAM-1 ELISA
Levels of soluble VCAM-1 (sVCAM-1) were determined in EDTA-plasma by ELISA using a commercially available sVCAM-1 module kit (Bender MedSystems, Vienna, Austria). The protocol for this module kit was similar to the protocol used to measure sICAM-1, except for the dilutions of both the standard protein and the plasma samples. Dilutions of the sVCAM-1 standard protein were made to a range of 3.2 ng/ml to 100 ng/ml and plasma samples were applied at a dilution of 1:50. The maximum detectable value in our system was 5000 ng/ml. Plasma samples exceeding the maximal OD of the standard were set at 5000 ng/ml (n=4, all during HCMV infection).

Soluble E-selectin ELISA
Soluble E-selectin (sE-sel) in EDTA-plasma was studied using a commercially available ELISA kit (Bender MedSystems, Vienna, Austria). Microtiter wells precoated with a monoclonal antibody against human sE-selectin were incubated with an E-selectin standard (range 0.8 ng/ml – 50.0 ng/ml) or five-fold diluted plasma samples. In the next step, without washing, an anti-sE-selectin monoclonal antibody conjugated to HRP was added. After a colour reaction with TMB, the optical density was measured and the sE-selectin concentration in the samples could be calculated.

Detection of CEC and EC
CEC and EC in blood were analyzed as described recently [7]. In brief, mononuclear cells (MNC) were isolated by density centrifugation using Lymphoprep (Nycomed Pharma AS, Oslo, Norway). 1 x 10^5 MNC were cytocentrifuged on a slide.
<table>
<thead>
<tr>
<th>HCMV-pp65 antigenemia*</th>
<th>N</th>
<th>Group</th>
<th>primary</th>
<th>secondary</th>
<th>HCMV symptoms</th>
<th>vascular</th>
<th>rejection</th>
<th>vascular</th>
<th>steroid resistant</th>
<th>steroid sensitive</th>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>3</td>
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<td>12</td>
<td>2</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Median: 11 – 100</td>
<td>11</td>
<td>3</td>
<td>3</td>
<td>9</td>
<td>5</td>
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<td>5</td>
<td>10</td>
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<td></td>
<td>11</td>
<td>26</td>
<td>15</td>
<td>4</td>
<td>8</td>
<td>12</td>
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</tbody>
</table>

*No. positive granulocytes/50,000
The cytospots were stained by indirect immunofluorescence with the following antibodies: C10/C11 directed against HCMV pp65 and E1/1 2.3 directed to a 90kD cell surface antigen of endothelial cells [21]. 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 or EC/ml blood in 95% of all samples.

Statistics

The distribution of patients among the different groups was tested using contingency tables ($\chi^2$-test). Differences between two groups were analysed using the Mann-Whitney test. Multiple groups were analysed using non-parametric analysis of variance: Kruskal Wallis test. Dunn’s Multiple Comparison test was used as post test if $P<0.05$. Multiple regression analysis was performed using Systat software. Clinical symptoms, the occurrence of CEC, EC, and acute rejection episodes were analyzed as categorical variables and the severity of HCMV infection was analyzed as a continuous variable.

Results

Patients

Forty-six kidney transplant patients were included (total number of samples 274) and classified into groups 1 to 4, with no (n = 10), low (n=12), moderate (n=11) or high HCMV viral load (n=13), respectively (Table 1). Signs and symptoms of HCMV infection were only observed in patients with moderate or high viral load; 5/11 of group 3 and 10/13 of group 4 ($P<0.001$). They had at least one sign or symptom of otherwise unexplained fever, malaise, leukocytopenia, thrombocytopenia and elevated serum levels of liver enzymes. Of all patients, twenty-four patients experienced one or more rejection episodes before the study period of HCMV infection. The median time span from diagnosis of rejection to the onset of HCMV infection was 16.5 days (range 7 -73 days). The patients with acute rejection that preceded HCMV infection or occurred before the expected onset of HCMV infection (group 1) were equally distributed among the four groups ($P=0.45$)(Table 1). Twelve had interstitial rejection that recovered after treatment with methylprednisolone, eight patients had steroid resistant interstitial rejection, the remaining four patients had vascular rejection. Patients with vascular rejection were all in the moderate and high viral load groups.

vWF

Baseline levels were obtained before HCMV infection at 15 days post transplantation. In transplant recipients, the median vWF baseline levels were higher (median 275%, range 85 to 555%) than the standard of healthy controls (range 50 to 150%). No differences among the antigenemia groups were observed ($P=0.52$). In the transplant recipients without HCMV infection, the vWF plasma levels decreased during time. At 50 days after transplantation, i.e. the median time of maximum vWF levels in HCMV patients (group 2-4), the patients without HCMV infection (group 1) had a median decrease of 62.5% (range 266 to –24%)
A comparison of the kinetics in vWF levels and kinetics of HCMV antigenemia showed that maximum vWF levels were obtained a few days after maximum HCMV antigenemia (3-7 days) (Fig. 2). The increase of vWF during HCMV infection was determined by subtracting baseline levels from the maximum vWF levels. Although the median values showed a rise with increasing viral load, only in patients of group 4, vWF plasma levels were significantly higher than in patients without HCMV infection (P<0.01); (group 4: median increase 113%; range -34 to 308%) (Fig. 1A).

**Figure 1**
Changes in plasma levels of vWF, sICAM-1, sVCAM-1 and sE-sel during HCMV infection. Patients were divided into four categories with no (0), low (1-10 pp65 + PMN/50.000), moderate (11-100) and high (>100) HCMV viral load. *P<0.05 (high versus none), ** P < 0.01(high versus none).

**sICAM-1**
At 15 days post transplantation the mean level of sICAM-1 in our patients was 354 ng/ml (range 138 to 853 ng/ml), which was about twofold higher than in healthy individuals (range
130 to 297 ng/ml). Baseline values were not different between the four groups (P=0.71). In patients without HCMV infection baseline levels of sICAM-1 were elevated at 15 days post transplantation and remained elevated at 50 days (group 1: median increase 12 ng/ml, range - 136 to 323 ng/ml)(Fig. 1). The sICAM-1 plasma levels during HCMV infection were determined at the same timepoints as vWF levels and changes of sICAM-1 plasma levels were expressed in the same way as those of vWF. No significant differences in changes between the four patients groups were demonstrated (P=0.11) (Fig. 1B).

sVCAM-1
At 15 days post transplantation, values of sVCAM-1 were higher in patients (median 1859 ng/ml, range 991 to 3914 ng/ml) than in healthy individuals (normal range 675 to 1693 ng/ml). Between the four groups the baseline values were not different (P=0.38). The sVCAM-1 values remained at the same level at 50 days after transplantation (group 1 median decrease 11ng/ml, range 1459 to –425 ng/ml). During HCMV infection, sVCAM-1 plasma levels were measured at similar timepoints as vWF plasma levels. During HCMV infection patients with high HCMV viral load had sVCAM-1 plasma levels almost double that of patient without HCMV infection (group 4: median increase 1532.2 ng/ml; range -1531 to 3349 ng/ml) (P<0.05) (Fig. 1).

sE-selectin
In 10 patients of group 1 and 10 patients of group 4 sE-selectin levels were determined at 15 and 50 days post transplantation, respectively. In these groups, 4 patients were included who had acute rejection episodes before or during HCMV infection. At 15 days post transplantation sE-selectin plasma levels (median 46 ng/ml, range 6 – 101 ng/ml) were not different from healthy controls (median 52.8 ng/ml, range 23.0 – 79.2 ng/ml). Furthermore, no differences in changes at 50 days between patients with a high HCMV viral load and patients without HCMV infection were detectable (P=0.32) (Fig. 1).

CEC and EC in peripheral blood
In patients with HCMV infection and detectable CEC in peripheral blood (n=9) only vWF plasma levels were significantly higher than in HCMV patients without CEC (P<0.05)(Fig. 2A). The occurrence of EC in blood of patients (n=14) was related to increased levels of vWF as well (P<0.001)(Fig. 2D). The presence of CEC and/or EC in peripheral blood of patients was not associated with significantly altered levels of sVCAM-1 or sICAM-1 (CEC: Fig. 2B, C; EC: Fig. 2E, F).

Kinetics of soluble parameters and endothelial cells during HCMV infection
The time kinetics of soluble parameters for endothelial damage were demonstrated in a typical patient with HCMV infection. This patient had a primary HCMV infection without clinical signs and symptoms and was not treated with ganciclovir. No acute rejection episodes were diagnosed. The patient had a high HCMV antigenemia with a maximum of
2500 pp65⁺ PMNs/50.000 at day 40 after transplantation that rapidly reduced to 480 pp65⁺ PMNs/50.000 at day 47 and 1 pp65⁺ PMN/50.000 at day 54. The maximum level of vWF was obtained at day 47 (414%) and sVCAM-1 levels were elevated from day 40 until day 54 (approx. 5000 ng/ml). After day 54 both levels started to decrease. The highest levels of CEC in blood were observed at day 47 (10.2 CEC/ml). This was in accordance with the maximum levels of vWF and sVCAM-1 and was slightly delayed compared to the HCMV antigenemia (7 days). This patient had no endothelial cells during HCMV infection. Although levels of sICAM-1 fluctuated during the infection, no correlation with HCMV antigenemia values was observed (Fig. 3).

Figure 2
Changes in plasma levels of vWF (A, D), sICAM-1 (B, E) and sVCAM-1(C, F) of patients without (open symbols) or with (closed symbols) cytomegalic endothelial cells (A-C) and endothelial cells (D-F) in peripheral blood. ** P<0.01 and ***P<0.001.
Signs and symptoms

There were no significant differences in changes of plasma levels of vWF, sICAM-1 or sVCAM-1 between patients with and those without HCMV associated clinical signs or symptoms (Fig. 4). Seven of 10 patients treated with ganciclovir had clinical symptoms.

Figure 3

vWF, sVCAM-1, sICAM-1 plasma levels in a patient with a primary HCMV infection. The bars indicate cytomegalic endothelial cells (CEC) per ml blood. This patient had no acute rejection episodes, no clinical signs and symptoms and was not treated with ganciclovir.

Acute rejection episodes

To study the effect of preceding acute rejection episodes and HCMV infection a different classification was made with the following categories: no acute rejection and no HCMV infection, acute rejection only, HCMV infection only, and acute rejection episodes before HCMV infection. A non-parametric ANOVA on these four categories revealed significant differences in changes of vWF plasma levels (P<0.01). Between the individual categories, patients with HCMV infections and preceding acute rejection episodes had a significantly higher increase of vWF plasma levels (median increase 87.5, range -143 to 308) than patients without acute rejection and no HCMV infection or patients with HCMV infection only (Fig. 5A). Analysis between vascular rejection, steroid resistant interstitial rejection or interstitial rejection responding to steroid treatment showed no differences in the increase of vWF (data not shown). The non-parametric ANOVA on changes of sVCAM-1 plasma levels showed that the four categories were different (P = 0.0215), though differences between individual categories did not reach significance (Fig. 5B). No significant differences were observed for sICAM-1 levels in plasma among the four categories (Fig. 5C).
**Correlation**

The changes of sVCAM-1 and sICAM-1 levels at 50 days post transplantation were correlated ($r = 0.50$, $P<0.001$). vWF showed just no correlation with one of these adhesion molecules (sVCAM-1: $r = 0.28$, $P=0.06$ and sICAM-1: $r = 0.27$, $P=0.07$).

**Figure 4**

Changes in plasma levels of vWF (A), sICAM-1 (B) and sVCAM-1(C) of patients without (open symbols) or with (closed symbols) HCMV associated symptoms.

**Multiple logistic regression**

Multiple regression analysis identified vWF and sVCAM-1 as independent parameters of the severity of infection ($P<0.001$). Only vWF was showed to be an independent predictor for the occurrence of CEC ($P<0.01$), EC ($P<0.01$), and acute rejection episodes ($P<0.05$). vWF, sVCAM-1 or sICAM-1 were not predictive for clinical symptoms of HCMV infection.

**Discussion**

In the present study we show in patients, with active HCMV infections after renal transplantation, that increased plasma levels of vWF were correlated to the occurrence of cytomegalic endothelial cells (CEC) and non-infected endothelial cells (EC) in the blood stream. Increases in vWF plasma levels as well as that of VCAM-1 were related to the severity of HCMV infection as measured with the HCMV antigenemia test. The most pronounced increases of both markers were observed in patients with both HCMV infection and preceding acute transplant rejection episodes. Changes in plasma levels of sICAM-1 or sE-sel did not relate the severity of infection or HCMV clinical symptoms.
With this study we focused on short-term effects of cytomegalovirus infection with a follow-up of 125 days after transplantation. Interestingly, the correlation of vWF and sVCAM-1 with the severity of the infection may reflect both an inflammatory response as well as damage to the endothelial surface of blood vessels. Of both markers, sVCAM-1 merely reflects endothelial activation.

Figure 5
Changes in plasma levels of vWF (A), sVCAM-1(B) and sICAM-1(C) of patients: without HCMV infection or acute rejection (none), with acute rejection but no HCMV infection (rej), with HCMV infection but no acute rejection (HCMV) and with both HCMV infection and acute rejection (HCMV/rej). * P<0.05 (HCMV/rej versus none).
It is released by shedding of the membrane bound form [13]. VCAM-1, via interaction with ligand VLA-4, has an important role in leukocyte adhesion and infiltration in tissues [23,24]. Biopsies have shown that both during acute rejection and HCMV infection the expression of VCAM-1 on several types of endothelial cells is increased, frequently at sites of infiltrated inflammatory cells [25-28]. vWF can be released after endothelial damage and to a lesser extent after an inflammatory stimulus [8]. The strong relation between increased levels of vWF and CEC, i.e. HCMV infected and detached endothelial cells, or EC indicates that endothelial damage is associated with HCMV infection. Thus, based on the findings of elevated vWF levels in blood, we postulate that during HCMV infection systemic low level vascular damage occurs.

Clinical signs and symptoms did not correlate with levels of soluble adhesion molecules and vWF. A possible explanation could be that early diagnosis and rapid treatment of patients with ganciclovir mitigates the occurrence of clinical symptoms. However, in the present study 70 percent of all patients treated with ganciclovir developed one or more clinical symptoms and had moderate to high HCMV antigenemia (Table 1). Therefore, in the present study it is doubtfull that treatment with ganciclovir has masked a possible correlation between clinical symptoms and levels of soluble markers.

In two earlier small-scale studies increased levels of sICAM-1 or sVCAM-1 were reported during HCMV disease [14,15]. Unfortunately, no information was given about the viral load or HCMV antigenemia in these studies. The present study, performed in a larger group of HCMV patients with an infection ranging from mild to severe could not confirm the findings about sICAM-1 and sVCAM-1. Thus, although most patients with clinical symptoms also had a high viral load, this did not result in a profile of increased inflammation and endothelial damage than patients without HCMV associated clinical signs and symptoms.

At 15 days after transplantation but before HCMV antigenemia became positive, the baseline of vWF in our patient group was three-fold higher than in healthy individuals. Also sICAM-1 and sVCAM-1 were elevated. Apparently, these patients had pre-existing endothelial activation or damage, that could be caused by many factors, such as surgery and subsequent reperfusion of the transplanted kidney [29], vascular injury due to hypertension or dialysis, or vascular damage by cyclosporin A [9,10]. In the absence of further complications like acute rejection or HCMV infection the baseline of vWF decreased during time.

Recently, in a study of the occurrence of endothelial cells in blood, we observed increased endothelial damage during HCMV infection, when this infection occurred after preceding episodes of acute rejection [7]. In accordance with our precious study, the present study demonstrated the increased release of both vWF and sVCAM-1 during HCMV infection in patients who experienced acute rejection before infection (Fig. 5). The question remains what the possible nature of this relationship is. We observed no correlation between the time span from diagnosis of acute rejection to the onset of HCMV infection (median time 16.5 days, range 7 -73 days) and the increase of vWF (r=0.027, P=0.91). Thus, patients having HCMV infection one week after acute rejection or patients having HCMV infection two month after acute rejection showed similar increases in vWF levels during HCMV infection. This argues against a continuous endothelial damage during HCMV infection induced by acute transplant rejection, otherwise we would expect a decline during time.
During acute transplant rejection but also during chronic transplant rejection, we postulated that levels of vWF are increased [11,12]. Also sVCAM-1 is reported to be elevated during acute rejection, although other studies could not find elevated levels of sVCAM-1 in serum of patients with rejecting grafts [30-33]. The endothelial damage induced by acute rejection is supposed to originate from the transplanted graft. In contrast, HCMV induces a systemic infection that may involve many organs. It seems likely to us that the occurrence of acute rejection episodes, effectuated at the allograft, sensitizes the endothelial surface, probably of the host, to HCMV induced damage. Therefore it would be interesting to know the balance between levels of VWF and sVCAM-1 released by endothelial cells from the graft or from the host.

The enhancement of endothelial injury during HCMV infection after acute rejection may have consequences in the long run. Both acute rejection episodes and HCMV infection are identified as risk factors for chronic transplant dysfunction [34-37]. With this study we demonstrate enhanced endothelial damage shortly after transplantation. No information is available about the underlying mechanisms of the enhancement between acute transplant rejection and HCMV associated vascular damage. The elevated baseline levels of both vWF and soluble adhesion molecules indicate a low level of ongoing vascular activation and damage by multiple factors, that might predispose these patients to chronic graft dysfunction. In vitro experiments have proven that HCMV infected endothelial cells do not express VCAM-1 and MHC class II molecules, not even after cytokine induction such as TNF-α and IFN-γ [38]. Nevertheless HCMV infected cells evoke a powerful proliferative and cytolytic response in T cells from HCMV seropositive donors. The cytokines produced in this response induce the expression of adhesion molecules and MHC class II at non-infected surrounding endothelial cells [39]. In turn, these cells become susceptible to alloreactive T cells. When this happens in a graft, a few HCMV infected endothelial cells in a graft may already trigger alloreactivity [40].

Experimental studies of CMV infection in rats that underwent either allogeneic or syngenic lung transplantations have shown that cytomegalovirus infection and acute rejection enhanced each other. Moreover, this was reflected in higher expression levels of the adhesion molecules ICAM-1 and VCAM-1, their counterligands LFA-1 and VLA-4 and MHC class II [41]. The role of CMV in the development of chronic graft dysfunction is extensively studied in rat models transplanted with aorta or cardiac allografts. In this model, infection with CMV enhanced multiple consecutive steps resulting in the development of chronic rejection. This included endothelial adhesion molecule expression, influx of inflammatory cells and smooth muscle cell proliferation [42-44]. In allograft recipients the occurrence of CMV infections is associated with development of chronic rejection [45,46]. In the present study we describe a cumulative effect of two events harmful for the endothelial surface early after transplantation. Whether the combination of multiple risk factors enhance the incidence of chronic rejection needs to be investigated.

In conclusion, both vWF and sVCAM-1 plasma levels were increased only during severe HCMV infection and were predictive for the viral load. Increased vWF plasma levels were related to the occurrence of CEC, thus confirming HCMV induced vascular damage. The combination of HCMV infection and preceding acute rejection caused the highest increase in
vWF and sVCAM-1 plasma levels, indicating that acute transplant rejection episodes before onset of HCMV infection enhance HCMV induced vascular damage at the moment of infection. The enhanced susceptibility for acute endothelial injury may indicate that these patients also have an increased risk for chronic transplant dysfunction. Further studies are needed to proof, whether this assumption may be true.

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