CONCISE COMMUNICATION

Uninfected and Cytomegalic Endothelial Cells in Blood during Cytomegalovirus Infection: Effect of Acute Rejection

A. M. Kas-Deelen,1 E. F. de Maar,2 M. C. Harmsen,1 C. Driessen,1 W. J. van Son,2 and T. H. The1

Departments of 1Clinical Immunology and 2Nephrology, University Hospital Groningen, Groningen, The Netherlands

After transplantation, human cytomegalovirus (HCMV) infections can cause vascular damage to 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, the appearance and numbers of endothelial cells (ECs) in blood of 54 kidney transplant recipients were investigated in a prospective clinical study. Two types of endothelial cells were identified: cytomegalic ECs (CECs) were detected in patients with moderate or high HCMV antigenemia, and uninfected ECs were observed in patients with and without HCMV infection. The incidence of either CECs, ECs, or the combination of both was associated with HCMV-related clinical symptoms (P < .01). Remarkably, the occurrence of rejection episodes before HCMV infection was an important risk factor for the occurrence of ECs in blood (ECs, CECs, or both) during HCMV infection (P < .001).

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 (ECs) that could be involved in viral dissemination [2, 3]. Infected ECs can occasionally detach from the basal membrane, enter the bloodstream, and 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 ECs (CECs) 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 CECs varies between different immunosuppressed populations [5, 7]. CECs in peripheral blood have been found to be associated with high virus 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 ECs in blood, HCMV disease symptoms, and transplantation rejection episodes. Isolated mononuclear cell fractions on cytopsots were studied by immunocytologic staining for the presence of ECs. Further investigation with markers for different stages of HCMV infection was used to examine whether, in addition to CECs, ECs 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 HCMV antigenemia for <1–2 weeks were excluded (n = 12), as were 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 antithymocyte globulin (bioMérieux, Lyon, France) in case of steroid-resistant rejection. Vascular rejection episodes were treated with antithymocyte globulin and plasmapheresis. Patients were monitored twice a week for HCMV antigenemia. The HCMV antigenemia test was done according the procedure recently reviewed for standardization [1].

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

Blood samples to study the occurrence of ECs were obtained before HCMV infection at ~15 days after transplantation and weekly after the first positive HCMV antigenemia test result. This was continued until the HCMV antigenemia test was negative (n = 32) or showed <5 pp65-positive granulocytes/50,000 cells (n = 12). Blood samples of patients without HCMV infection (n = 10) were studied at ~15, 40, 50, and 60 days after transplantation.

CECs in peripheral blood were analyzed according to a quantitative method as described elsewhere [4, 9]. Briefly, heparinized blood samples were obtained by venipuncture. The mononuclear
cell fraction was isolated by density gradient centrifugation by means of Lymfoprep (Nycomed Pharma, Oslo). On each slide, 1 × 10^5 mononuclear cells were cytocentrifuged. For each sample, a variable number of cytocdots was analyzed, depending on the concentration of mononuclear cells per milliliter of blood. Four cytocdots were analyzed if there were > 1.5 × 10^6 mononuclear cells/mL of blood; otherwise, 6–8 cytocdots were analyzed. The number of analyzed slides represented a detection limit of 20 CECs/mL of 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 ECs from blood [9]. This correction factor was included in the calculation. The following monoclonal antibodies were used for staining of the cytocdots: C10/C11 directed against HCMV pp65 and E1/1 2.3 directed to a 90-kDa cell surface antigen of ECs [10]. ECs were stained with E13 directed against HCMV immediate-early proteins (Seralab, Sussex, UK). Fixation was with 1% paraformaldehyde, followed by indirect immunofluorescence double-staining with fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate-positive cells only.

Statistical analyses were done with contingency tables (χ² test), nonparametric Mann-Whitney test, or nonparametric analysis of variance (Kruskal-Wallis) for differences in distribution between groups, differences between 2 groups, and differences between multiple groups, respectively.

Results

Fifty-four patients were included in this study (32 men, 22 women; 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 4 groups, depending on the highest HCMV antigenemia measurement (pp65-positive granulocytes/50,000 cells): none, low (1±10), moderate (11±100), or high (>100). Thirteen of 16 patients in the group with high antigenemia and 5 of 12 patients with moderate antigenemia had clinical symptoms, such as fever, malaise, leukocytopenia, thrombocytopenia, and elevated levels of liver enzymes. Twenty-eight patients had 1 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 rejection. Episodes of vascular rejection were associated with high virus load: none occurred in the groups with no or low antigenemia, compared with 5 in the groups with moderate or high antigenemia. Patients with 1 or more rejection episodes were equally distributed among groups (P = .41; table 1). Two distinct types of ECs in peripheral blood were observed: late-stage-infected CECs and uninfected ECs. We never observed ECs in immediate-early or early stages of HCMV infection. Both CECs and ECs were detectable in blood at or just after the maximum HCMV antigenemia peak. After maximum HCMV antigenemia, ECs could be detected for a longer time than could CECs. In 3 of 10 patients without HCMV infection, ECs were demonstrated. Two of these patients had ECs at 15 days after 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 ECs were detected during HCMV antigenemia and never before HCMV infection.

CECs were detected in 11 (25%) of 44 patients with HCMV infection (figure 1A): 8 of 16 patients with high antigenemia and 3 of 12 patients with moderate antigenemia (groups 3 and 4; figure 1A). Concentrations of CECs ranged from 0.11 to 30.26/mL of blood (median, 0.89; figure 1C). In 4 patients with high antigenemia, CECs were detected at various times during HCMV antigenemia. ECs were observed in all patient categories independent of the severity of infection (figure 1B). The concentrations of ECs ranged from 0.17 to 114.05/mL of blood (median, 2.62; figure 1D).

Patients with rejection episodes had ECs in blood (CECs, CECs, or both) during HCMV infection more often (66.7%) than did patients without rejection (15.0%; P < .001). The detection of ECs was not significantly related to the type of rejection. A tendency could be observed to higher frequencies of CECs or ECs in patients with a more severe type of rejection (6/13 patients with steroid-sensitive interstitial rejection vs. 12/15 patients with vascular rejection or steroid-resistant interstitial rejection).

Patients with CECs had significantly more HCMV-associated clinical symptoms (81.8%) than did patients without CECs (27.3%; P < .01). Eleven (68.8%) of 16 HCMV patients with
ECs had HCMV-associated symptoms, compared with 7 (25%) of the remaining 28 patients ($P < .01$). Fourteen patients with moderate or high antigenemia, of whom 7 had detectable CECs, were treated with ganciclovir. Ten of 14 patients had clinical symptoms and were treated with ganciclovir.

**Discussion**

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

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

Release of uninfected ECs has been described for several abnormalities with vascular injury, such as sickle cell anemia [11]. These authors describe ECs in circulation in healthy persons [11]. With the procedure used in our study, we were not able to detect ECs in the blood of healthy persons (data not shown).

The occurrence of ECs in peripheral blood was closely related to active HCMV infection, even though these cells are not in-
fected. 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 ECs observed during HCMV infection were negative for CD45, making it unlikely that they were bone marrow derived.

Detection of CECs, ECs, or both in HCMV patients was strongly related to the occurrence of earlier rejection episodes. CECs 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 tumor necrosis factor-α. Binding of tumor necrosis factor-α 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 ECs originate from preexisting endothelial lesions in the transplanted graft, probably enhanced by HCMV. Especially during vascular rejection, damage is directed at the endothelium. In our study, 4 of 5 patients with vascular rejection had ECs 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 antithymocyte globulin treatment (unpublished data) and represented a more severe form of interstitial rejection. Furthermore, because of sampling error in taking biopsies, vascular lesions at different sites in the graft could be missed.

In conclusion, the occurrence of CECs, ECs, 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 ECs. 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 has cumulative effects at the endothelial cell surface, which may predispose these patients toward chronic graft failure.

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References