CHAPTER 9

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

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The Journal of Infectious Diseases 2000; 181:721-724

ABSTRACT

After transplantation cytomegalovirus (CMV) 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 CMV 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 CMV antigenemia, and uninfected ECs were observed in patients with and without CMV infection. The incidence of either CECs, ECs, or the combination of both was associated with CMV-related clinical symptoms ($P < 0.01$). Remarkably, the occurrence of rejection episodes before CMV infection was an important risk factor for the occurrence of ECs in blood (ECs, CECs, or both) during CMV infection ($P < 0.001$).

9.1 INTRODUCTION

Human cytomegalovirus (CMV) 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 CMV-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 CMV 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 CMV infection after renal transplantation we studied the relationship between the appearance of distinct ECs in blood, CMV disease symptoms, and transplantation rejection episodes. Isolated mononuclear cell fractions on cytospots were studied by immunocytologic staining for the presence of ECs. Further investigation with markers for different stages of CMV 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.

9.2 PATIENTS AND METHODS

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

No CMV prophylaxis, such as ganciclovir, acyclovir or hyperimmune gamma globulin, was given. Fourteen patients received ganciclovir because of clinical symptoms associated with rising CMV antigenemia values. Blood samples to study the occurrence of ECs were obtained before CMV infection at about 15 days after transplantation and weekly after the first positive CMV antigenemia test result. This was continued until the CMV antigenemia test was negative (n = 32) or showed less than 5 pp65-positive granulocytes/50.000 cells (n = 12). Blood samples of patients without CMV infection (n = 10) were studied at about 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 x 10^5 mononuclear cells were cytocentrifuged. For each sample, a variable number of cytospots was analyzed, depending on the concentration of mononuclear cells per milliliter of blood. Four cytospots were analyzed if there were $\leq$1.5 x 10^6 mononuclear cells/ml of blood; otherwise 6-8 cytospots 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 cytospots: C10/C11 directed against CMV pp65 and E1/1 2.3 directed to a 90-kDa cell surface antigen of ECs [10]. ECs were stained with E13 directed against CMV immediate-early proteins (Seralab, Sussex, UK). Fixation was with 1% paraformaldehyde, followed by indirect immunofluorescence double-staining with fluorescein isothiocyanate or tetramethyl rhodamine (Southern Biotechnology Associates, Birmingham, AL) for endothelial-specific markers and CMV antigens, respectively. CECs and ECs were determined by counting double-positive cells or fluorescein isothiocyanate-positive cells only.

Statistical analyses were done with contingency tables (χ^2 test), non-parametric Mann-Whithney test or non-parametric analysis of variance (Kruskal-Wallis) for differences in distribution between groups, differences between 2 groups and differences between multiple groups, respectively.

9.3 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 four groups depending on the highest CMV 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 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 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 ($P = 0.41$; table 9.1).

Table 9.1 Acute rejection and human cytomegalovirus (CMV) infection among patients after kidney transplantation.

group, by CMV pp65 antigenemia* (*n*)	no. of patients with CMV infection		no. of patients with CMV symptoms	no. of rejection episodes		
					interstitial	
	prim	sec		vascular	st. res	st. sen
none: 0 (10)	0	0	0	0	1	3
low: 1-10 (16)	0	16	0	0	1	6
moderate: 11-100 (12)	3	9	5	2	5	1
high: > 100 (16)	10	6	13	3	3	3
total (54)	13	31	18	5	10	13

prim = primary
sec = secondary
st. res = steroid-resistant
st. sen = steroid-sensitive
* No. of pp65-positive granulocytes/50.000 cells.

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 CMV infection. Both CECs and ECs were detectable in blood at or just after the maximum CMV antigenemia peak. After maximum CMV antigenemia, ECs could be detected for a longer time than could CECs. In 3 of 10 patients without CMV infection, ECs were demonstrated. Two of these patients had ECs at 15 days post transplantation, which was shortly after a rejection episode. The other patient experienced neither rejection nor CMV infection. Remarkably, in the patients with CMV infection, all ECs were detected during CMV antigenemia and never before CMV infection.

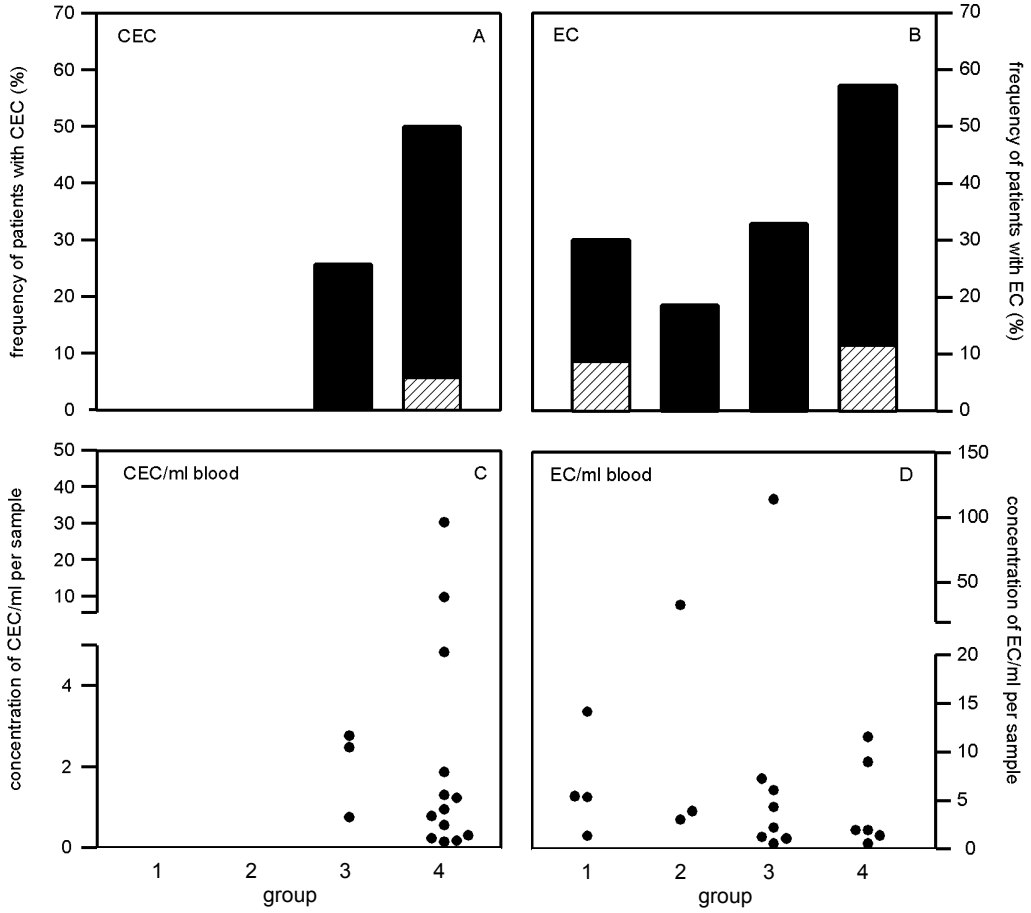
CECs were detected in 11 (25%) of 44 patients with CMV infection (figure 9.1a): 8 of 16 patients with high antigenemia and 3 of 12 patients with moderate antigenemia (groups 3 and 4; figure 9.1a). Concentrations of CECs ranged from 0.11 to 30.26/ml (median 0.89; figure 9.1c). In 4 patients with high antigenemia, CECs were detected at various times during CMV antigenemia.

ECs were observed in all patient categories independent of the severity of infection (figure 9.1b). The concentrations of ECs ranged from 0.17 to 114.05/ml blood (median 2.62; figure 9.1d).

Patients with rejection episodes had ECs in blood (ECs, CECs, or both) during CMV infection more often (66.7%) than patients without rejection (15.0%; $P < 0.001$). 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 of patients with vascular rejection or steroid resistant interstitial rejection).

Patients with CECs had significantly more CMV-associated clinical symptoms (81.8%) than did patients without CECs (27.3%; $P < 0.01$). Eleven (68.8%) of 16 CMV patients with ECs had CMV-associated symptoms, compared with 7 (25%) of the remaining 28 patients ($P < 0.01$). 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.

Figure 9.1 **Frequencies (A, B) and concentrations (C, D) of cytomegalic endothelial cells (CEC; A, C) and endothelial cells (EC; B, D) in peripheral blood of patients with cytomegalovirus (CMV) infection. Frequency is the number of patients/group with cells at any time during infection. Hatched portions of bar are patients with CMV infections; solid portions are patients with both preceding acute rejection episodes and CMV infection. Groups 1-4: no, low, moderate or high antigenemia, respectively.**



9.4 DISCUSSION

This study demonstrates that the appearance of CECs, as well as of ECs is related to CMV antigenemia levels, as well as to CMV-associated symptoms. Intriguingly, patients with acute rejection episodes and CMV 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 CEC 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 CMV 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 CMV treatment was given influenced not only the course of CMV 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 individuals (data not shown).

The occurrence of ECs in peripheral blood was closely related to active CMV 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 ECs observed during CMV infection were negative for CD45, making it unlikely that they were bone marrow derived.

Detection of CECs, ECs or both in CMV patients was strongly related to the occurrence of earlier rejection episodes. CECs were mainly observed in patients with high CMV 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-□. Binding of tumor necrosis factor-□ could stimulate the CMV immediate early promoter/enhancer region and thus enhance the infectivity of that cell by CMV [13].

It is also possible that the ECs originate from preexisting endothelial lesions in the transplanted graft, probably enhanced by CMV. Especially during vascular rejection, damage is directed at the endothelium. In our study 4 of 5 patients with vascular rejection had ECs during CMV infection. According to the Banff criteria [8] only

arterial involvement is a criterion for vascular 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 represent 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 CMV antigenemia and CMV-associated clinical symptoms. Transplant rejection mechanisms and CMV infection have a cumulative effect on the release of endothelial cells. Many studies have shown that both CMV 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.

9.5 REFERENCES

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