

# Circulating Endothelial Cells Are a Novel Marker of Cyclosporine-Induced Endothelial Damage

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**Abstract**—Microvascular endothelial cells play a key role in transplant immunology. They are also important targets for calcineurin inhibitors. We recently demonstrated elevated numbers of circulating endothelial cells in renal transplant recipients with and without rejection in comparison with healthy controls. Because these patients received either cyclosporine or tacrolimus, we speculated that endothelial damage from calcineurin inhibitors might be responsible for these findings. In the present study, we tested the hypothesis that treatment with calcineurin inhibitors leads to an increase in circulating endothelial cells. We studied 57 renal transplant recipients: 19 on a calcineurin inhibitor-free immunosuppressive regimen and 38 patients on a standard immunosuppressive regimen, including cyclosporine, and matched them for age and serum creatinine. Endothelial cells were isolated from peripheral blood with anti-CD-146-coated immunomagnetic Dynabeads™ and were counted by fluorescence microscopy. Patients with cyclosporine therapy had elevated numbers of circulating endothelial cells (median 26, range 12 to 82 cells/mL) compared with healthy controls (median 6, range 0 to 82 cells/mL;  $P < 0.001$ ). Patients without calcineurin inhibitor treatment had significantly lower cell numbers (median 12, range 0 to 32 cells/mL;  $P < 0.003$ ) and were not significantly different from normal, untreated controls. In conclusion, renal transplant recipients who do not receive calcineurin inhibitors have significantly lower numbers of circulating endothelial cells than their age- and creatinine-matched counterparts who receive these drugs. We suggest that elevated numbers of circulating endothelial cells indicate damage from calcineurin inhibitors in renal transplant recipients and that circulating endothelial cells are a novel marker of endothelial damage. (*Hypertension*. 2003;41[part 2]:720-723.)

**Key Words:** cyclosporine ■ endothelium ■ transplantation ■ vascular diseases

The long-term prognosis of renal grafts has not improved as much as 1-year graft survival rates despite remarkable progress in immunosuppressive therapy. Therefore, factors other than immune-mediated damage have been implicated.<sup>1</sup> Several lines of evidence suggest that the toxicity of calcineurin inhibitors plays a key role in propagating and aggravating chronic graft dysfunction. Firstly, the withdrawal or reduction of calcineurin inhibitors in patients with chronic allograft nephropathy<sup>2</sup> or patients with histological evidence of cyclosporine toxicity<sup>3</sup> has been shown to preserve<sup>2</sup> or even improve renal function.<sup>3</sup> Secondly, trials using a standard immunosuppressive regimen without calcineurin inhibitors have reported lower creatinine levels after 6 months.<sup>4</sup> Finally, a number of deleterious effects of calcineurin inhibitors, such as intrarenal vasoconstriction and stimulation of fibrogenic cytokines<sup>5</sup> have been documented in vitro. These observations have been associated with damaging effects of calcineurin inhibitors on endothelial cell function.<sup>3,4,5</sup>

However, markers of calcineurin inhibitor-mediated endothelial damage are not available at present. Cyclosporine

blood levels do not correlate with endothelial damage, and features of cyclosporine toxicity in biopsies are nonspecific. Circulating endothelial cells (CECs) have been used as a marker of endothelial damage in a variety of vascular disorders.<sup>6</sup> Recently, we demonstrated elevated numbers of CECs in renal transplant recipients with vascular rejection (A. Woywodt, M. Schroeder, W. Gwinner, M. Mengel, B. Maess, M. Jaeger, A. Schwarz, H. Haller, and M. Haubitz, unpublished data, 2002).<sup>7</sup> Because all of these patients received cyclosporine or tacrolimus, the aim of the present study was to investigate whether calcineurin inhibitors may be responsible for the elevated numbers of CECs and whether this approach can be used to assess endothelial cell damage in renal transplant recipients.

## Methods

Fifty-seven renal transplant recipients were investigated. Nineteen patients were on a calcineurin inhibitor-free immunosuppressive regimen. Thirty-eight patients who received cyclosporine served as matched controls regarding age and renal function. This study was

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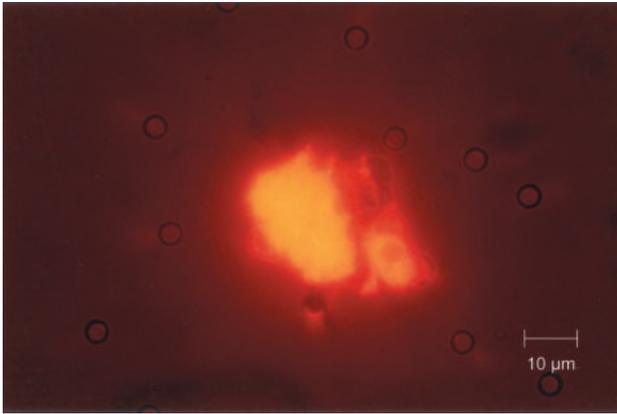
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**Figure 1.** Circulating endothelial cells (UEA-1 stain) isolated from a renal transplant patient.

approved by the local institutional review board, and informed consent was obtained from each patient. Twenty-one healthy volunteers (ages 23 to 71 years, median 43 years) served as controls. Patients with acute rejection were not included.

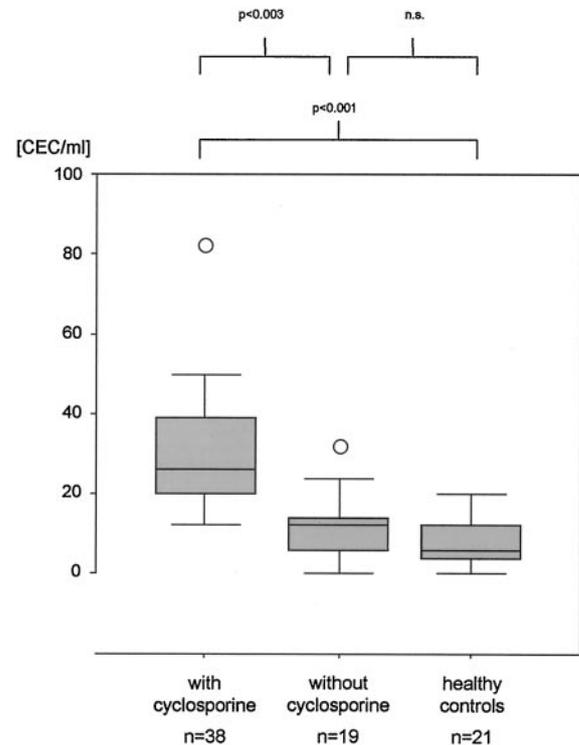
Peripheral blood was obtained before biopsy and 12 hours after the last dose of cyclosporine in the cyclosporine group. CECs were isolated within 6 hours with anti-CD-146-coated immunomagnetic Dynabeads (Dyna) as previously described<sup>7</sup> and counted in a Nageotte chamber by fluorescence microscopy. Human umbilical vein endothelial cells were diluted in blood from healthy volunteers to serve as controls. Staining with von Willebrand factor, CD 31, and Ulex europaeus lectin-1 (UEA-1) was used to prove the endothelial origin of these cells.

The results are given as medians and ranges. Mann-Whitney *U* test was used for comparison of cell numbers of patients and normal controls. Paired Wilcoxon test was used for comparison of cell numbers of patients without calcineurin inhibitors and patients with cyclosporine treatment (the 2 patients with cyclosporine, matched with 1 patient without calcineurin inhibitors, were averaged). Spearman rank test was used for calculation of a possible correlation between cyclosporine trough levels and CEC numbers.

## Results

We investigated 57 patients after renal transplantation. The male-to-female ratio was comparable in both groups. The mean age was not significantly different between the 2 groups. All patients had stable renal function. Thirty-eight of these patients received cyclosporine and 19 were without. The immunosuppressive regimen consisted of steroids and mycophenolate mofetil (MMF); 7 patients in the cyclosporine-free group were on azathioprine. Mean cyclosporine plasma levels were 142 ng/mL (range 84 to 288). The cyclosporine group had a mean plasma creatinine concentration of 129  $\mu$ mol/L (range 62 to 245), whereas the plasma creatinine in the patients without cyclosporine was 143  $\mu$ mol/L (range 65 to 214). The morphology of circulating endothelial cells was described in detail elsewhere (A. Woywodt, M. Schroeder, W. Gwinner, M. Mengel, B. Maess, M. Jaeger, A. Schwarz, H. Haller, and M. Haubitz, unpublished data, 2002). Briefly, the cells were round or oval in shape and 20 to 50  $\mu$ m in size, carried more than 5 beads, and were UEA-1-positive (Figure 1).

Patients on cyclosporine had significantly elevated numbers of CECs compared with healthy controls ( $P < 0.001$ )



**Figure 2.** Circulating endothelial cells in renal transplant patients without calcineurin inhibitors compared with patients with cyclosporine and with healthy controls.

(Figure 2). Cell numbers did not correlate with age, gender, or underlying kidney disease. In patients with cyclosporine, no significant correlation between cell numbers and cyclosporine trough levels could be shown. In contrast, CEC numbers were significantly lower in patients who did not receive a calcineurin inhibitor compared with patients with cyclosporine treatment (12, range 0 to 32 cells/mL, versus 26, range 12 to 82 cells/mL;  $P < 0.003$ ) and did not differ from cell numbers in healthy controls (6, range 0 to 20 cells/mL, Figure 2).

## Discussion

The main finding of this study is that renal transplant recipients without calcineurin inhibitors have significantly lower numbers of CECs than their matched counterparts who receive cyclosporine. In addition, the numbers of CECs in the calcineurin-free group were not significantly different from those of healthy controls. We therefore suggest that elevated numbers of CECs in renal transplant recipients reflect endothelial damage from calcineurin inhibitors (A. Woywodt, M. Schroeder, W. Gwinner, M. Mengel, B. Maess, M. Jaeger, A. Schwarz, H. Haller, and M. Haubitz, unpublished data, 2002).

Our findings are in accord with several studies reporting potentially deleterious effects of calcineurin inhibitors on endothelial morphology and function. In vitro, cyclosporine inhibits endothelial cell replication and induces the formation of cytoplasmic vesicles and nucleolar changes.<sup>8</sup> Moreover, inhibition of respiratory chain enzymes<sup>9</sup> and enhanced vascular permeability<sup>10</sup> have been documented in response to

cyclosporine. Cyclosporine also impairs the production of nitric oxide in animal models<sup>11</sup> and humans.<sup>12</sup>

The possible origin of CECs in renal transplant recipients also deserves attention. Traditionally, studies of endothelial damage have focused on the glomerulus and its vicinity. Yet recent research has elucidated immune-mediated endothelial damage in peritubular capillaries with deposition of Cd4 as a feature of chronic allograft nephropathy and predictor of long-term graft loss.<sup>13</sup> Therefore, elevated numbers of CECs in renal transplant recipients (A. Woywodt, M. Schroeder, W. Gwinner, M. Mengel, B. Maess, M. Jaeger, A. Schwarz, H. Haller, and M. Haubitz, unpublished data, 2002) may be of peritubular origin and predict chronic graft loss. Alternatively, CECs may also originate from recipient endothelium beyond the boundaries of the renal allograft. It has been postulated previously that cyclosporine-induced endothelial toxicity, reflected by numbers of CECs, may contribute to arteriosclerosis and vascular disease in renal transplant recipients.<sup>14</sup> Additional studies, such as attempts to demonstrate the Y chromosome in circulating endothelial cells from female recipients of male grafts, are therefore necessary to determine the origin of CECs in renal transplant recipients. However, we assume that our cells are not part of a repair process because endothelial progenitor cells are CD 146-negative.

Whether or not tacrolimus causes as many endothelial side effects as cyclosporine is unknown. Accordingly, we matched our calcineurin inhibitor-free patients to those receiving cyclosporine. However, a pattern of small-vessel injury indistinguishable from that caused by cyclosporine has been observed in biopsies from renal transplant recipients who receive tacrolimus.<sup>15,16</sup> Moreover, Solez et al<sup>17</sup> found no histopathological difference between renal transplant recipients who were randomized to either tacrolimus or cyclosporine. In analogy to findings in patients on cyclosporine,<sup>2</sup> Weir and co-workers showed that 50% of renal transplant recipients receiving tacrolimus benefited from a dose reduction, in terms of graft function.<sup>2</sup> Therefore, one may speculate that endothelial toxicity is equally present in patients with cyclosporine and tacrolimus.

Few, if any, laboratory markers of ongoing vascular damage caused by calcineurin inhibitors are currently available. The likelihood and extent of endothelial damage do not correlate well with the serum levels of these drugs. Biopsy samples fail to reflect the true extent of endothelial damage because histological changes seem to occur late in the process of toxicity and are nonspecific and easily missed because of sampling error. Cyclosporine toxicity is therefore difficult to diagnose, and novel markers of these effects are awaited. Tiemann et al<sup>18</sup> reported elevated levels of von Willebrand factor (vWF) and tissue factor pathway inhibitor in heart transplant patients. However, the pathophysiological implications of these findings are difficult to assess because vWF has prothrombotic and probably atherogenic properties, whereas tissue factor pathway inhibitor is antithrombotic and probably antiatherogenic. Specificity is another issue because elevated levels of vWF have been reported in a variety of other disorders<sup>19,20</sup> and depend on renal function.<sup>21</sup> We and others have previously established the peripheral blood endothelial

cell count as a sensitive and specific marker of vascular disease.<sup>6,7,22</sup> The present study suggests that the number of CECs is also a novel marker of endothelial damage from calcineurin inhibitors.

### Perspectives

In summary, we have shown that treatment with cyclosporine is associated with an increase in circulating endothelial cells. These circulating endothelial cells may reflect endothelial cell damage by cyclosporine and are a promising marker of endothelial toxicity caused by calcineurin inhibitors. Long-term studies with correlation of CECs and graft function may further corroborate the clinical significance of our findings. A study of patients who receive calcineurin inhibitors for a variety of other disorders would also be worthwhile because normal cell numbers in that group would point to the graft as the origin of circulating endothelial cells in our patients. Moreover, further studies of the cell phenotype may help to elucidate the pathogenesis of endothelial damage. The measurement of circulating endothelial cells is a novel tool for evaluating endothelial cell damage in patients.

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