Cyclosporin A Suppresses Proliferation of Endothelial Progenitor Cells: Involvement of Nitric Oxide Synthase Inhibition

Long Yang¹, Xin-Chun Yang¹, Jun-Ke Yang¹, Ying-Hua Guo², Fang-Fang Yi¹, Qian Fan¹ and Xiu-Lan Liu¹

Abstract

Objective To investigate the effects of the potent immunosuppressive agent cyclosporin A (CsA) on the proliferation of human endothelial progenitor cells (EPCs) and endothelial nitric oxide synthase (eNOS) expression in EPCs.

Methods and Results The EPCs were obtained from cultured mononuclear cells, which were isolated from the peripheral blood of healthy adults, and stimulated with CsA (10 μg/mL) in the presence or absence of either vascular endothelial growth factor (VEGF; 50 ng/mL) or L-arginine (1 mM). To explore the effect of different concentrations of CsA alone on EPC proliferation, some cells were treated with CsA in a series of final concentrations ranging from 0 to 10 μg/mL. Cell proliferation and apoptosis were determined, respectively, by the Cell Counting Kit-8 assay and terminal deoxynucleotidyl transferase-mediated nick end labeling staining. The expression of eNOS was assayed by reverse transcription-polymerase chain reaction analysis while nitric oxide (NO) generation was detected using the Griess method. The effects of CsA on EPC proliferation, apoptosis, and eNOS/NO production were dose dependent in the concentration ranging from 0.1 μg/mL to 10 μg/mL. Treatment with VEGF (50 ng/mL) significantly promoted EPC proliferation and eNOS/NO production, which were completely abrogated by pre-incubation with CsA (10 μg/mL). The supplement of L-arginine (1 mM) promoted NO production that enhanced EPC proliferation and attenuated the effect of CsA on EPC proliferation and apoptosis.

Conclusion CsA significantly inhibited proliferation, eNOS mRNA expression and NO production of human EPCs, in a dose-dependent manner.

Key words: immunosuppressive drugs, stem cells, cell proliferation, apoptosis, nitric oxide synthase

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Introduction

The calcineurin inhibitor cyclosporin A (CsA) is a potent immunosuppressive agent that has formed the pharmacologic cornerstone of solid organ transplantation. Although pharmacologic studies of CsA have focused primarily on T cell responses, vascular injury has generally been considered a common important factor for all types of CsA-induced organ damage. The observation of multiple endothelial effects (1-5) indicates that endothelial cell toxicity is a feature of CsA-induced vascular injury.

Vascular endothelial progenitor cells (EPCs) are the precursors of mature endothelial cells, and contribute to postnatal neovascularization and re-endothelialization (6-10). About 26% endothelial cells of a new vessel are derived from EPCs (11) and play a significant role in angiogenesis and endothelial functions. We have drawn a hypothesis that EPC damage is induced by CsA, which may contribute to endothelial toxicity and vascular injury during CsA treat-
Cell isolation and culture

The activation of EPCs is regulated by numerous cytokines and the most important family of cytokines implicated in the neovascularization processes is the vascular endothelial growth factor (VEGF) family. It has been shown that VEGF plays an important role in regulating EPC migration, homing, adhesion, proliferation, tubule formation, and differentiation (12-16). CsA may differentially inhibit multiple steps in VEGF-induced angiogenesis, including the decrease of cell proliferation in human microvascular endothelial cells and human umbilical vein endothelial cells (1, 17).

Previous studies have demonstrated that endothelial nitric oxide synthase/nitric oxide (eNOS/NO) is a significant regulator of angiogenesis. The absence of eNOS in mice has revealed impaired ischemia-induced angiogenesis and reduced EPC mobilization (18, 19). Estrogen was shown to increase the number of EPCs by its ability to induce NO (20). The oxidized low-density lipoprotein (oxLDL) inhibits eNOS activity and down-regulates eNOS expression, leading to a conspicuous decrease in NO generation, which may promote EPC apoptosis and poor overall function (21). eNOS also contributes to the effect of VEGF in the improvement of EPC proliferation and function (18, 22, 23). Furthermore, the reduction of NO induced by CsA is critical to vascular dysfunction of patients who have received an organ transplant (24) and L-arginine, the substrate of NO synthesis, ameliorates CsA-induced vascular dysfunction (24-26).

Thus, we hypothesize that eNOS/NO is involved in the effects of CsA on EPC biology. In this study, we aim to investigate the effects of CsA on proliferation, eNOS gene expression, and NO production in cultured human peripheral blood EPCs.

Methods

Cell isolation and culture

The ex vivo expansion of EPCs was performed as described previously (6). In brief, mononuclear cells (MNCs) were isolated from peripheral blood drawn from healthy young volunteers by density gradient centrifugation. The cells were then plated on human fibronectin-coated (Gibco, Grand Island, NY, USA) culture dishes and maintained in M199 media (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), recombinant human vascular endothelial growth factor (VEGF; Cytolab, Rehovot, Israel), recombinant human fibroblast growth factor-basic (CytoLab), recombinant human epidermal growth factor (CytoLab), recombinant human insulin-like growth factor-I (CytoLab), penicillin, streptomycin, heparin, and ascorbic acid. After 4 d in culture, nonadherent cells were removed by washing the culture with phosphate-buffered saline (PBS), and a new medium was applied and replaced every 2 d.

Characterizations of EPCs

After culture for 7 d, the cells were subjected to flow cytometric analysis to examine the expression of CD34 (Becton & Dickinson, Mountain View, CA, USA), CD133 (Miltenyi, Bergisch Gladbach, Germany), and VEGFR2 (R&D, Minneapolis, MD, USA). The cells were incubated for 30 minutes at 4°C with monoclonal mouse anti-human antibodies against CD34, CD133, and VEGFR2. The isotype-matched antibodies served as the control. Thereafter, the cells were quantitatively analyzed by a fluorescence-activated cell sorter (Becton & Dickinson).

The binding of fluorescein isothiocyanate-labeled Ulex europaeus agglutinin I (FITC-UEA-I, 10 μg/mL; Sigma, St. Louis, MO, USA), a lectin specific for human endothelium, and uptake of 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labelled acetylated low-density lipoprotein (Dil-acLDL, 2.4 μg/mL; Molecular Probes Inc, Eugene, OR, USA) were measured to determine EPCs (6, 27). The cells were incubated with 10 μg/mL Dil-acLDL at 37°C for 4 hours and were fixed with 2% paraformaldehyde for 10 minutes. After washing, the cells were incubated with 10 μg/mL FITC-UEA-1 at 4°C for 1 hour. The incorporation of Dil-acLDL and binding of FITC-UEA-1 was detected with a confocal microscope (Leica Microsystems AG, Wetzlar, Germany). The nuclei were stained with 6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI, Sigma) in order to assess the rate of positive-stained cells. Numerous EPCs were evaluated by counting four randomly selected high-power fields (200×) in three wells in blinded fashion.

Treatment of EPCs

The cells were divided into the following six groups depending on the different treatments administered: control group (without agent); VEGF group (VEGF 50 ng/mL for 48 hours); CsA (Sigma) group (CsA 10 μg/mL for 48 hours); CsA+VEGF group (VEGF 50 ng/mL for 48 hours after pre-treated with CsA 10 μg/mL for 1 hour); L-arginine (Arg; Sigma) group (Arg 1 mM for 48 hours); and CsA + Arg group (both 10 μg/mL CsA and 1 mM Arg for 48 hours). There was no significant change in medium pH with the addition of agents. Another set of experiment was performed to explore the effect of CsA alone at different concentrations ranging from 0 to 10 μg/mL on EPC proliferation, apoptosis, and eNOS/NO production.

Proliferation assay

After culture for 7 d, adherent cells were digested with 0.25% trypsin and cultured with 0.5% FBS-M199 in 96-well culture plates at 2×10^4 cells per well for 24 hours. The medium was replaced with fresh 0.5% FBS-M199. The cells were treated by the aforementioned process. At 24 hours and 48 hours, the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used to assay cell proliferation according to the manufacturer’s instructions. Cells in each group were counted blindly at four randomly selected fields (200×) per well (six wells of each group) at the time points of 0 hours, 24 hours, and 48 hours after incubation with agents in order to assess the change of cell number in each


**Results**

**Characteristics of EPCs**

The total MNCs were isolated and cultured for 7 d, resulting in spindle-shaped, endothelial cell-like morphology as described previously (6). The EPCs were characterized as adherent cells, double positive for Dil-acLDL uptake and FITC-UEA-I binding (93.66±0.96)% (n=3, cell from 3 independent experiments). Analysis of the surface marker phenotype of the cells isolated in this fashion revealed that the positive rates of CD34, CD133, and VEGFR2 were (33.35±3.76)%, (20.61±3.36)%, and (68.83±7.25)%, respectively (n=3).

CsA inhibits proliferation capacity of EPCs

The treatment with CsA decreased EPC proliferation in a dose-dependent manner (Fig. 1). After intervention with agents, at the time points of 24 hours and 48 hours, CCK-8 assay and cell counting showed that CsA (10 μg/mL) notably suppressed EPC proliferation. VEGF (50 ng/mL) markedly promoted EPC proliferation which was abrogated by pretreated with 10 μg/mL CsA (Tables 1, 2). L-arginine (1 mM) enhanced EPC proliferation and attenuated the inhibitory effect of CsA on EPC proliferation (Tables 1, 2).

CsA increases apoptosis of EPCs

The treatment with CsA increased EPC apoptosis in a dose-dependent manner (Fig. 2). Co-incubation with L-arginine (1 mM) and CsA (10 μg/mL) resulted in a lower apoptotic rate as compared with incubation with CsA alone (Fig. 2).

CsA decreases eNOS mRNA and NO production in human EPCs

At the concentrations above 100 ng/mL, the CsA treatment markedly decreased eNOS mRNA expression (Fig. 3) and NO production (Fig. 4) in a dose dependent manner. Treatment with CsA (10 μg/mL) in the presence and absence of VEGF (50 ng/mL) decreased eNOS mRNA expression and NO production, and there was no significant differ-

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**TUNEL assay for measuring apoptosis**

For in situ detection of apoptotic cells, adherent cells were fixed with 2% paraformaldehyde. Then, the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay was applied according to the manufacturer’s instructions (Roche, Penzberg, Germany). The nuclei were counterstained with DAPI. TUNEL-positive cells were examined in a blinded fashion in four randomly-selected fields (200x) per well (six wells of each group) and the percentage of cells was calculated.

**Reverse transcription-polymerase chain reaction (RT-PCR) analyses for eNOS**

The total RNA was extracted using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription from total RNA was performed using SuperScript III (Invitrogen). The cDNA was analyzed by semiquantitative PCR under nonsaturating conditions. Two specific primers were used to identify and amplify eNOS (sense primer: 5’- CCTGAGACGGCCTGGC-3’; antisense primer: 5’- CCTAACATCTGGCACAGTCCCTTA-3’). The RT-PCR-amplified samples were visualized at 2% agarose gels using ethidium bromide. Each mRNA band was normalized with internal reference glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA. A band of interest was analyzed by the scan analysis software (AlphaEaseFC, Alpha Innotech, San Leandro, CA, USA) and expressed as a ratio of GAPDH mRNA.

**Determination of NO generation**

NO is unstable, but it forms stable end products, namely, nitrite and nitrate. Hence, the content of nitrite and nitrate is a suitable index of NO generation. The EPC culture medium was harvested and the level of nitrite was measured with the Griess Reagent Kit (NO2/NO3 Assay Kit-C II; Dojindo) according to the manufacturer’s instructions.

**Statistical analysis**

The results are expressed as mean ± SEM. Statistical significance was evaluated by means of a one-way analysis of variance (ANOVA; least significant difference, LSD test; Dunnett T3 test) or t-test. A value of p<0.05 was considered to denote statistical significance.
Table 1. Effects of CsA, VEGF, and L-arginine on EPC Proliferation (450 nm Light Absorbance; ± x̄)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>VEGF</th>
<th>CsA</th>
<th>CsA+VEGF</th>
<th>Arg</th>
<th>CsA+Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.52±0.08</td>
<td>0.88±0.14 *</td>
<td>0.34±0.04 *</td>
<td>0.36±0.09 *</td>
<td>0.79±0.17 *</td>
<td>0.64±0.14 *</td>
</tr>
<tr>
<td>48</td>
<td>0.57±0.08</td>
<td>1.07±1.11 *</td>
<td>0.21±0.04 *</td>
<td>0.24±0.06 *</td>
<td>0.96±0.16 *</td>
<td>0.70±0.16 *</td>
</tr>
</tbody>
</table>

*p<0.01, ^p<0.05 vs control group; "p<0.01 vs VEGF group; "p<0.01 vs CsA group; n=7. VEGF: vascular endothelial growth factor. CsA: cyclosporin A. Arg: L-arginine.

Table 2. Effects of CsA, VEGF, and L-arginine on EPC Number (x̄ ± x)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>VEGF</th>
<th>CsA</th>
<th>CsA+VEGF</th>
<th>Arg</th>
<th>CsA+Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>60.58±8.18</td>
<td>62.50±10.16</td>
<td>59.38±5.06</td>
<td>59.12±3.61</td>
<td>60.35±7.20</td>
<td>61.24±5.76</td>
</tr>
<tr>
<td>24h</td>
<td>65.67±6.25</td>
<td>77.54±9.64 *</td>
<td>40.38±3.92 *</td>
<td>43.17±4.57 *</td>
<td>76.62±6.01 b</td>
<td>72.12±5.16 d</td>
</tr>
<tr>
<td>48h</td>
<td>72.17±4.62</td>
<td>99.71±13.87 *</td>
<td>32.15±3.27 *</td>
<td>34.67±3.28 *</td>
<td>95.96±4.45 *</td>
<td>84.50±5.15 *</td>
</tr>
</tbody>
</table>

*p<0.01, ^p<0.05 vs control group; "p<0.01 vs VEGF group; "p<0.01 vs CsA group; n=6. VEGF: vascular endothelial growth factor. CsA: cyclosporin A. Arg: L-arginine.

Discussion

An immunosuppressant is necessary to prevent graft rejection after a solid organ transplant. However, it is also known to have significant side effects, including endothelial toxicity.

The growth of new microvessels (or angiogenesis), and the re-endothelialization of impaired endothelium, is now considered to be a critical biologic process involving EPCs. The effect of CsA treatment on EPC number is controversial from previous studies. Davies et al (28) indicated that CsA, even at concentrations lower than therapeutic levels (10-200 ng/mL), potently decreased cell number by inhibiting EPC proliferation. However, Miriuka et al (29) reported that CsA decreased the EPC number only at a very high concentration (10 μg/mL).

In the present study, we demonstrated that CsA exerted a potent inhibitory effect on EPC proliferation at the concentrations of 100-10,000 ng/mL, and the effect was dose-dependent. At the dose of 10 ng/mL, CsA obviously failed to alter the EPC proliferation. The decrease of cell number is due to the effect of CsA treatment on both the inhibited proliferation and the increased apoptosis.

Although there are some differential effects among calcineurin inhibitors on cell activity (30-33), it is not completely clear whether other calcineurin inhibitors, such as tacrolimus and sirolimus, have the same effect as CsA on regulating EPC proliferation and apoptosis. To our knowledge, the only study on the effect of CsA and another calcineurin inhibitor (tacrolimus) on EPC activity, reported by Miriuka and colleagues (29) showed that the treatment with CsA but not with tacrolimus induced a significant reduction in cultured EPC number. Future studies are necessary to elucidate the differential effect of any of the other calcineurin inhibitors.

The effect of CsA on EPC number in vivo is controversial according to the results of previous studies. Davies and colleagues (28) showed that CsA treatment deceased colony forming units of EPCs (it represents the number of EPCs) from peripheral blood of swine received either cardiac transplantation or sham operation. Inversely, the work of Wang et al (34) showed that the number of circulating EPCs was markedly increased by the CsA treatment in mice of ischemic limb models by way of inhibiting the activity of CD26/DPP IV (dipeptidylpeptidase IV) system. This contributed to the increased concentrations of stroma-derived factor-1α (SDF-1α) and stem cell factor (SCF). Interestingly, Wang et al (34) also found that before ischemic stress, treatment with CsA made no significant difference in the blood levels of any of the cytokines and EPC number, indicating that CsA alone does not interfere with cytokine catabolism and EPC mobilization. The exact reason is not clear why CsA treatment causes controversial results as reported in the aforementioned studies. Further studies are needed to clarify these points.

One of the major angiogenic growth factors is VEGF, which may induce migration, proliferation, and tube formation in EPCs (12, 13, 15, 16). The CsA may differentially inhibit multiple steps in VEGF-induced angiogenesis including decreasing the proliferative capacity in mature endothelial cells (1, 17). The effect of CsA on regulating the proliferative capacity in the presence of VEGF in EPCs is not clear. We found that pre-treatment with CsA (10 μg/mL) completely abrogated EPC proliferation induced by VEGF, which indicated that CsA-induced endothelial toxicity is at least partly due to the inhibition of VEGF functions.

Along with the inhibition of eNOS mRNA expression and NO production by CsA, we found that EPC apoptosis was obviously increased, and cell proliferation was potently inhibited. Treatment with L-arginine, the substrate of NO syn-
Figure 2. The effect of CsA on EPC apoptosis. A: Control, B: 0.1 μg/mL CsA, C: 0.5 μg/mL CsA, D: 10 μg/mL CsA, E: 50 ng/mL VEGF, F: 10 μg/mL CsA+VEGF, G: 1 nM Arg, H: 10 μg/mL CsA+Arg. Representative photographs are given. Green, TUNEL positive; blue, staining of nuclei with DAPI. Bar chart shows that the effect of CsA on EPC apoptosis is dose dependent, and co-incubation with CsA and L-arginine completely abrogated the effect of CsA on EPC apoptosis (\( p<0.01 \) vs Control group; \( p<0.01 \) vs 0.1 μg/mL CsA group; \( p<0.05 \) vs 0.5 μg/mL CsA group; \( p<0.01 \) vs 10 μg/mL CsA+VEGF group; \( p<0.01 \) vs 10 μg/mL CsA group; n=6).
The effect of CsA treatment on the expression of eNOS gene in EPCs. The mRNA of eNOS were amplified by RT-PCR; samples were visualized on 2% agarose gels using ethidium bromide. The bar chart shows semiquantitative analysis of band intensity of RT-PCR. The ratio of integrated optical density (IOD) from eNOS mRNA band to GAPDH mRNA band was calculated. The data were shown as percentage of the data from control group. The effect of CsA on the expression of eNOS gene is dose dependent. Stimulation with VEGF promoted eNOS expression which was completely abrogated by CsA. Treatment with L-arginine failed to change eNOS expression (ap<0.01 vs Control group; bp<0.01 vs 0.1 μg/mL CsA group; cp<0.05 vs 0.1 μg/mL CsA group; dp<0.05 vs 0.5 μg/mL CsA group; ep<0.01 vs 50 ng/mL VEGF group; n=3).

The effect of CsA treatment on the production of NO generation in EPCs. The production of NO was assayed by the Griess method. The data were shown as percentage of control group. Bar chart shows that the effect of CsA on NO production is dose dependent, and co-incubation with CsA and L-arginine significantly increased NO production as compared with CsA alone. Stimulation with VEGF potently promoted NO production which was completely abrogated by CsA (ap<0.01 vs Control group; bp<0.05 vs Control group; cp<0.01 vs 0.1 μg/mL CsA group; dp<0.05 vs 0.1 μg/mL CsA group; ep<0.01 vs 10 μg/mL CsA group; n=6).

Previous studies in stem/progenitor cells and mature endothelial cells indicated that VEGF induces the expression of resulting in the promotion of stem/progenitor cells differentiation into endothelial cells and increasing endothelial cell proliferation through the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway (35-37). Rafiee et al (17) showed that CsA significantly inhibits VEGF-induced migration, proliferation, and tube formation of human intestinal microvascular endothelial cells (HIMEC), and the effect of CsA on signaling events following VEGF-induced HIMEC activation is mediated partly through the inhibition of ERK1/2 activation. These studies
suggested that CsA may prevent VEGF-induced eNOS expression by inhibiting the ERK1/2 activity.

In conclusion, we showed that CsA inhibits proliferation, eNOS mRNA expression and NO production of cultured human EPCs from peripheral blood, in a concentration-dependent manner.

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References


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