Cyclosporine increases ischemia-induced endothelial progenitor cell mobilization through manipulation of the CD26 system

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Cyclosporine mobilizes endothelial progenitor cells (EPCs) from bone marrow (11, 26). The mobilization of EPCs for regeneration of ischemic tissues, such as stroma-derived factor-1α (SDF-1α)/CXCL 12, which serves as a chemoattractant for human CD34+ cells and stem/progenitor cell populations. Previous reports have demonstrated that immunosuppressed organ transplant patients exhibit lower CD26/DPP IV serum activity compared with healthy individuals (18, 19). It is possible that cyclosporine treatment lowers the CD26/DPP IV activity in peripheral blood. The attenuated CD26/DPP IV enzyme activity may subsequently contribute to an increase in the circulating SDF-1α concentration, which is closely related to the numbers of circulating EPCs mobilized from the bone marrow and the amount of EPC-associated angiogenesis in ischemic tissues. However, the effect of CsA on angiogenesis is still controversial (8, 17, 22, 25, 31). Some studies have shown that CsA is an antiangiogenic medication (22, 31). This study sought to test the hypothesis that short-term treatment with cyclosporine, an immunosuppressant, stimulates EPC mobilization after ischemic stress through manipulation of the CD26/DPP IV system.

METHODS

Animal Studies

Male C57BL/6 wild-type mice (Jackson Laboratory, Bar Harbor, ME) or bone marrow-reconstituted FVB mice (BMT→Wild mice) underwent severe hindlimb ischemia surgery under general anesthesia. Animals were treated subcutaneously with CsA (15 mg·kg⁻¹·day⁻¹) (15, 30) and/or diprotin-A (a DPP IV antagonist, 5 μmol twice daily). CsA solution (Sandimmune, Novartis, NJ) was a microemulsion preconcentrate (50 mg/ml) containing polyoxyethylated castor oil (650 mg/ml) and absolute alcohol 32.9% (vol/vol) in a sterile ampoule. Immediately before the subcutaneous injection, the CsA solution was diluted 20 times in normal saline. Control animals were injected with normal saline. Treatments began 4 days before surgery and stopped as indicated after surgery in the different experiments. The study protocol was reviewed and approved by the Committee on Ethics for Animal Experiments, Chang Gung Memorial Hospital Faculty of Medicine, and the experiments were conducted according to the guidelines of the American Physiological Society.
Cell Culture

Human aortic smooth muscle cells were purchased from Smartec Scientific (Cascade Biologics) and grown in 231 medium with smooth muscle cell growth supplement plus 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO2. Cells used for the experiments were in the third to fifth passages and were 80% confluent. After cells were washed with PBS, the medium was replaced with serum-free medium with or without CsA (300 and 500 μg/l), and cells were incubated and harvested 24 h later. Supernatant was harvested at 0, 6, 12, and 24 h for further analysis.

Assessment of White Blood Cells in Peripheral Blood

At different time points (at baseline and 6, 12, 24, and 48 h), blood samples were obtained via a tail vein. The total number of white blood cells (WBCs) was assessed with a Neubauer hematocytometer (Fisher Scientific).

Fluorescence-Activated Cell Sorting Analysis

The fluorescence-activated cell sorting FACSCaliber flow cytometer (Becton Dickinson) was used to assess EPC mobilization from the bone marrow. For the mouse studies, whole blood was obtained by cardiac puncture at the indicated time points following hindlimb ischemia. Antibodies including phycoerythrin (PE)-conjugated anti-c-kit (clone 2B8; Biolegend), FITC-conjugated anti-CD31 (clone MEC13; Biolegend), PE-conjugated anti-sca-1 (clone E13-161.7; Pharmingen), and FITC-conjugated anti-KDR (Av'Ostix12x1; Pharmingen) were used to identify putative EPCs. The EPCs were considered to be from the mononuclear cell population and were double positive for c-kit and CD31 (12, 14) or sca-1 and KDR (27). Circulating EPCs were quantified by enumerating c-kit+/CD31+ and sca-1+/KDR+ cells. In addition, the quantity of CD26+ cells in the peripheral blood was estimated using an FITC-conjugated anti-mouse CD26 antibody.

Measurement of Cytokines

Whole blood, with EDTA as an anticoagulant, was centrifuged to isolate the plasma, which was then stored at −80°C for future analysis. Plasma SDF-1α concentrations were measured with a mouse SDF-1α ELISA kit (R&D Systems). The plasma concentrations of vascular endothelial growth factor (VEGF), stem cell factor (SCF), and granulocyte-colony stimulating factor (G-CSF) (R&D Systems) were assessed at different time points following surgery.

Real-Time Quantitative PCR Systems

Mouse gastrocnemius muscles were harvested at the indicated time points after hindlimb ischemia was induced. Tissues were homogenized with the MagNA Lyser (Roche Diagnostics, Penzberg, Germany). Total cellular RNA was extracted using the Trizol reagent. Real-time quantitative PCR (Q-PCR) was performed with the LightCycler TaqMan Master Mix (Roche Diagnostics). For each reaction, 1 μg of total RNA served as a template. For amplification, primer pairs specific for mouse SDF-1α (sense primer, 5'-CTG TGC CCT TCA GAT TTG TG-3'; antisense primer, 5'-TAA TTT CCG GTC AAT GCA CA-3') and SCF (sense primer, 5'-AGC TTG TCA TCA ACG GGA AG-3'; antisense primer, 5'-TTT GAT GTT AGT GGG GTC TG-3') were used. The reaction sequence included denaturation at 10 min at 95°C before 45 cycles of denaturation for 10 s at 95°C, annealing for 30 s at 60°C, and extension for 1 s at 72°C. Thermal cycling and collection of fluorescence data were done in a Roche LightCycler instrument.

Measurement of CD26 Activity

CD26 peptidase activity of the plasma was measured in 96-well microplates using the chromogenic substrates Gly-Pro-p-nitroanilide (Gly-Pro-pNA; Sigma, St Louis, MO) as previously reported (21). Peptidase activity was estimated as picomoles per minute. Prolyl-lyase activity was determined by measuring the amount of pNA formed in the supernatant at 405 nm. In the 96-well flat-bottom plate, 100 μl of plasma per well were incubated at 37°C with 1.5 mM Gly-Pro-pNA in 100 μl of PBS buffer (pH 7.4) containing 10 mg/ml BSA. Absorbance was measured at 405 nm on a microplate reader (Olympus MR, MRXII; DYNEX Technologies) at 3-min intervals for a total of 60 min, and the number of picomoles of pNA formed was calculated by comparison to a pNA standard curve. The results were plotted as picomoles of pNA versus time, giving a measure of DPP IV activity expressed as picomoles per minute. The peak pNA production rate was used for analysis between groups. Tests were run using six separate samples (n = 3 for each sample); cell-free blanks and substrate-free blanks were run in parallel.

Gelatin Zymography

Hindlimb muscle tissues were harvested at different time points and frozen at −80°C. These tissues were pulverized under liquid nitrogen and incubated for 1 h at 4°C with 60 μl of extraction buffer (10 mM sodium phosphate buffer, pH 7.2, containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 0.1% NaN3). After extensive vortexing and centrifugation at 13,000 rpm for 5 min, the protein content of the supernatants was determined. For the zymographic analysis of gelatinase activity, samples of arterial extracts were electroblotted on a 10% Tris-glycine gel with 0.1% gelatin (Novex; SanverTECH). The gel was renatured for 30 min at room temperature in 2.5% Triton X-100 and developed overnight at 37°C in 50 mM Tris-HCl buffer (pH 8.8) containing 5 mM CaCl2 and 0.02% NaN3. Staining was performed using 0.1% Coomassie brilliant blue R-250, and destaining was performed using destaining solution (methanol-acetic acid-water, 4:5:1:4.5).

Bone Marrow Transplantation Model

Recipient FVB mice at 8 wk of age were lethally irradiated with a total dose of 900 rad. eGFP transgenic mice (FVB background) that ubiquitously expressed enhanced green fluorescent protein (eGFP) were used as the donors (Level Biotechnology, Taipei, Taiwan) (13). After irradiation, the recipient mice received unfractionated bone marrow cells (5 × 106) from the eGFP mouse by a tail vein injection. Eight weeks after the injection, surgery to induce hindlimb ischemia was performed. Repopulation by eGFP-positive bone marrow cells was measured by flow cytometry to be 95%.

Hindlimb Ischemia Model

Mice underwent surgery to induce severe unilateral hindlimb ischemia. The animals were anesthetized using isoflurane inhalation. Under sufficient anesthesia, the left external iliac artery and vein, the deep femoral arteries and veins, and the entire left superficial femoral artery and vein (from just below the deep femoral arteries to the popliteal artery and vein) were ligated, cut, and excised. To estimate limb perfusion 2 wk after ischemia induction, the ischemic (right)-to-normal (left) limb blood flow ratio was measured with a laser-Doppler imager (MoorLDi-20M; Moor Instruments). After two laser Doppler images were recorded, the average perfusion for the ischemic and nonischemic limbs was calculated on the basis of colored histogram pixels.

In Vivo Angiogenesis Assay

Growth factor-free reconstituted basement membrane proteins (Matrigel; Becton Dickinson, Franklin Lakes, NJ), previously thawed at 4°C, were used for the in vivo angiogenesis assay. Briefly, 400 μl of reconstituted Matrigel were injected subcutaneously in the control and CsA-treated bone marrow-reconstituted mice (n = 6 for each group). Fourteen days after the Matrigel injection, plugs were removed and processed for histological analysis. The EPCs within the

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plug were recognized by eGFP+CD146+. Angiogenesis was evaluated in a blinded manner by considering at least three different sections per Matrigel plug; each section was 100 μm from the next. The total number of EPCs over the entire area of the Matrigel plug was measured and expressed as the number of EPCs [per high-power field (HPF)].

Confocal Immunofluorescent and Histological Analysis

Two weeks following the induction of hindlimb ischemia in the bone marrow-reconstituted mice (BMThGFp-><wild mice), tissues were harvested for confocal immunofluorescent and histological analysis. Tissue neovascularization was assessed in 5-μm frozen sections of the gastrocnemius muscles from the ischemic limbs. EPCs were stained with antibodies directed against von Willebrand factor (vWF; DAKO) and eGFP (Chemicon). EPC density was estimated by counting eGFP+vWF+ cells under a HPF (×400) in at least 10 different cross sections from different animals.

Statistical Analysis

Data were analyzed using one-way analysis of variance or t-test. When statistically significant effects were found, Tukey’s test was performed to isolate the differences between groups. Student’s t-test was used when appropriate. A P value <0.05 was considered significant. All data are means ± SE.

RESULTS

Cyclosporine Increases the Number of Circulating EPCs

Around 6–24 h following artery ligation, the number of peripheral WBCs significantly increased and then gradually returned to the baseline level 48 h later (Fig. 1A). To enumerate circulating putative EPCs, flow cytometry was performed with gating on the mononuclear cell population. There were no significant differences in peripheral blood total WBC count between the control and the CsA groups. Previously, we showed that mobilization of EPCs from the bone marrow peaked between 12 and 24 h after hindlimb ischemia was induced (28). Thus, in this study, EPC mobilization was estimated at 18 h after surgery. Compared with the control group, the CsA group displayed a significant increase in the number of circulating EPCs 18 h after hindlimb ischemic stress. Compared with the controls, the CsA group had a twofold increase in c-kit+CD31+ cells (Fig. 1B) and a fivefold increase in sca-1+KDR+ cells (Fig. 1C). Next, diprotin-A, a DPP IV inhibitor, was used to investigate the role of the DPP IV system in the effect of CsA. Diprotin-A alone had no effects on c-kit+CD31+ or sca-1+KDR+ EPC mobilization, probably due to a balanced effect of diprotin-A on inhibiting DPP IV activity in both bone marrow and peripheral blood (5, 28).

Fig. 1. Cyclosporine [cyclosporin A (CsA)] increases the number of circulating endothelial progenitor cells (EPCs). A: the number of peripheral white blood cells (WBCs) was enumerated in the control and CsA group at indicated time points after hindlimb ischemia was induced. B and C: EPC mobilization was estimated at 18 h after hindlimb ischemia was induced. Circulating EPCs were defined as c-kit+CD31+ cells (B) or sca-1+KDR+ cells (C). D and E: the effect of CsA on EPC mobilization was significantly blocked by diprotin-A. Values are means SE (n = 10 for each group). *P < 0.05; **P < 0.001 compared with controls. †P < 0.05; ‡P < 0.001 compared with CsA + Isch group. Isch, ischemia.
However, an intervention with diprotin-A significantly blocked the effect of CsA on EPC mobilization (Fig. 1, D and E).

Cyclosporine Modulates Blood Cytokine Levels in Response to Ischemic Stress

Concentrations of blood SDF-1α, VEGF, G-CSF, and SCF were measured at different time points following hindlimb ischemic stress. In the control group, SDF-1α concentrations transiently increased at 24 h (Fig. 2A). However, the levels of SDF-1α in the CsA group were three times higher than those in the control group at 6 and 12 h ($P < 0.001$). In the control group, SCF concentrations were transiently but insignificantly higher at 6 h (Fig. 2B). However, the levels of SCF in the CsA group were two times higher than those in the control group at 12 h ($P < 0.001$). The levels of VEGF and G-CSF remarkably increased within 24 h (Fig. 2, C and D). However, no significant differences were observed in the levels of VEGF or G-CSF between the CsA and control groups.

Expression of SCF and SDF-1 mRNA in Tissues Under Ischemic Stress

In vivo, the transcriptions of SCF and SDF-1 mRNA in the gastrocnemius muscle were quantified by Q-PCR. The data revealed that after creation of hindlimb ischemia, tissue SCF and SDF-1 mRNA transcription levels were not significantly upregulated through 48 h (Fig. 3, A and B). Furthermore, there was no significant difference in the amounts of SCF or SDF-1 mRNA transcription between the CsA and control groups.

Effect of Cyclosporine on the CD26/DPP IV System

The number of circulating CD26$^+$ cells was enumerated in mice with different interventions. Compared with the control group, ischemic stress or CsA treatment slightly but insignificantly lowered the number of CD26$^+$ cells in the peripheral blood. However, the number of circulating CD26$^+$ cells was significantly lower in the group undergoing ischemic stress and CsA treatment (Fig. 4A). Furthermore, plasma DPP IV activity was measured. Compared with the control group, hindlimb ischemic stress or CsA treatment lowered DPP IV activity with a synergistic effect for the intervention with both ischemic stress and CsA treatment (Fig. 4B).

Effect of Cyclosporine on the Matrix Metalloproteinase System

In vitro, vascular smooth muscle cells (VSMCs) are known to be able to synthesize and secrete matrix metalloproteinase (MMP)-2, MMP-9, and SDF-1α (29) and were used to investigate the effect of CsA on the MMP system. Our data showed that CsA had no significant effects on the activity of MMPs (Fig. 4C). Moreover, VSMCs secreted a significant amount of SDF-1α in a time-dependent manner. However, treatment with CsA did not significantly affect the secretion of SDF-1α by VSMCs (Fig. 4D).

The MMP system was also investigated in the hindlimb ischemia model. In vivo, the activities of MMP-2 and pro-MMP-9 were noted to have increased in time-dependent man-
Cyclosporine and EPC Mobilization

Main Findings

Cyclosporine in Angiogenesis

The effect of CsA on angiogenesis is still controversial. Most reports in the literature suggest that CsA is an antiangiogenic medication (22, 31). However, some studies have shown that CsA failed to prevent angiogenesis in vivo (22, 25). Furthermore, CsA has been suggested to promote the recurrence or development of cancer in experimental animal models through a proangiogenic effect (8, 17). Previous reports have shown that CsA can stimulate hematopoietic stem cell growth (23). It may be possible that the beneficial effect of CsA on cardiovascular disease occurs through bone marrow-derived EPCs as well. So far, the mobilization effect of CsA on EPCs is unappreciated and awaits further elucidation. Our study demonstrated that short-term CsA therapy increased the mobilization of EPCs, improved angiogenesis, and salvaged the ischemic limbs.

Importance and Mechanisms of EPC Mobilization

EPCs are primitive bone marrow cells with the capacity to proliferate, migrate, and differentiate into cells that line the lumen of blood vessels (1, 7, 26). Recent studies have provided increasing evidence that postnatal neovascularization does not consist exclusively of the sprouting of preexisting vessels but also involves a contribution of bone marrow-derived EPCs (9). Accordingly, an improved understanding of the regulation of EPCs may lead to new insights into ways to optimize the mobilization of EPCs for tissue regenerative therapy.

The contribution of bone marrow-derived EPCs to ischemic tissues was also investigated in this hindlimb ischemia model. An EPC was defined as eGFP+CD146+ cells. Compared with the controls, CsA treatment caused a twofold increase in the number of eGFP+CD146+ cells in the ischemic gastrocnemius muscle 14 days after ischemia had been induced (Fig. S5). Furthermore, on the 14th day after hindlimb surgery, CsA treatment significantly increased perfusion in the ischemic limb as estimated by laser-Doppler perfusion imaging (Fig. 5C) and lowered the spontaneous amputation rate compared with the controls (16.7 vs. 66.7%, P = 0.036; n = 18–23 for each group).

Discussion

The effect of CsA on angiogenesis is still controversial. Most reports in the literature suggest that CsA is an antiangiogenic medication (22, 31). However, some studies have shown that CsA failed to prevent angiogenesis in vivo (22, 25). Furthermore, CsA has been suggested to promote the recurrence or development of cancer in experimental animal models through a proangiogenic effect (8, 17). Previous reports have shown that CsA can stimulate hematopoietic stem cell growth (23). It may be possible that the beneficial effect of CsA on cardiovascular disease occurs through bone marrow-derived EPCs as well. So far, the mobilization effect of CsA on EPCs is unappreciated and awaits further elucidation. Our study demonstrated that short-term CsA therapy increased the mobilization of EPCs, improved angiogenesis, and salvaged the ischemic limbs.
known as CXCL12) plays an important role in mobilizing hematopoietic stem and progenitor cells from the bone marrow (4). Although redundancy exists in the majority of chemokine-receptor interactions, SDF-1/H9251 is one of a unique few in the chemokine subfamily of cytokines that binds to only one receptor, CXCR4, which is also expressed on EPCs (32).

Immunosuppressant Status and CD26/DPP IV Activity

Organ transplantation is the preferred treatment for most patients with chronic organ failure. Transplantation of the kidneys, liver, lungs, and heart offers an excellent opportunity for rehabilitation as recipients return to a more normal lifestyle. The discovery of CsA in the 1980s remarkably improved the short-term success rate of transplantation. CD26/DPP IV is a membrane-bound extracellular peptidase that specifically downregulates CXCL12/SDF-1α activation of CXCR4 receptor-presenting cells by cleaving the NH2-terminal dipeptide of CXCL12/SDF-1α. NH2-terminally truncated CXCL12/SDF-1α, lacking the first two amino acids, was reported to lack chemotactic activities. Thus CD26/DPP IV may provide a novel target for mobilizing stem/progenitor cells. Previous reports demonstrated that immunosuppressed organ transplant patients exhibit lower CD26/DPP IV serum activity compared with healthy individuals (18, 19). Organ transplantation can trigger the activity of D26/DPP IV in the immune cascade and lead to acute rejection of cardiac allografts in rat recipients. The effect of CsA on preventing organ rejection may also occur through lowering CD26/DPP IV activity. Our data support a direct effect of CsA on attenuating CD26/DPP IV activity in the peripheral blood. The attenuated CD26/DPP IV enzyme activity contributes to an increase in the circulating SDF-1α concentrations, which are closely related to levels of circulating EPC numbers mobilized from the bone marrow. The capacity of a DPP IV inhibitor, diprotin-A, to abolish this effect of CsA lends further supports to the associated mechanisms. Moreover, CsA appears to modulate DPP IV activity mainly in the peripheral blood. Further studies need to be performed to investigate the effects of CsA in the bone marrow in the future.

Mechanisms of Increases in SDF-1α and SCF Circulation Levels

CD26/DPP IV system. CD26 was originally described as a T-cell activation molecule but is now regarded as a non-lineage-specific antigen whose expression in hematopoietic cells is regulated by differentiation and activation (4). For example, CD26/DPP IV is not expressed on the surface of resting B or natural killer cells but is induced upon stimulation. It is also...
expressed on a specific set of T lymphocytes, where it is upregulated after activation. A variety of tissues, including endothelial cells, fibroblasts, and epithelial cells, express CD26 as well (4). In addition, CD26/DPP IV is present in a catalytically active soluble form in plasma.

Our data demonstrated that treatment with CsA had no significant effect on regulation of SDF-1 mRNA expression in ischemic tissues. The increased concentration of plasma SDF-1 after ischemic stress in the CsA treatment group substantially resulted from the effects of CsA on downregulating CD26/DPP IV activity in the plasma and decreasing CD26 cell numbers in the peripheral blood. CXCL12/SDF-1α chemoktracts human CD34+ cells and stem and progenitor cell populations, and it is considered an important component of the migration, homing, and mobilization of these important cells. Moreover, previous reports showed that the mobilization effect of G-CSF on hematopoietic stem/progenitor cells is also mediated by the CD26/DPP IV and CXCR4/SDF-1α pathways (2, 3).

MMP system. Purified MMP-2 and MMP-9 have been shown to cleave SDF-1α into a molecule, SDF-1α-(5–67), devoid of biological activity (6, 20). However, although peripheral mononuclear cells (PBMCs) accumulate over an area after tissue injury, the predominant accumulation of inactive MMP-9 precursor forms in PBMCs and/or the presence of natural MMP inhibitors may explain the absence of SDF-1α proteolysis by leukocyte-secreted MMP (24). Furthermore, our study revealed that, in vitro, MMP-2 and MMP-9 activities were not affected by CsA. In vivo, MMP-2 activity was not influenced by treatment with CsA. Furthermore, MMP-9 was not activated although there was significantly increased pro-MMP-9 activity in hindlimb tissues in the CsA-treated group compared with the controls. Understanding the effect of pro-MMP-9 on SDF-1α levels in the current study.

Previously, SDF-1α was shown to activate MMP-9 in the bone marrow and subsequently increase SCF levels by cleaving membrane-bound SCF into a soluble form. This phenomenon was not observed in local tissues after ischemic stress in our study. However, the increase of SCF in CsA-treated mice may be explained by other sources of SCF in response to an
increase in SDF-1α, such as in the bone marrow microenvironment (10).

**Perspectives and Significance**

This study demonstrates that CsA can exert its beneficial cardiovascular effects in part by altering CD26/DPP IV activity. In the era of cell-based therapeutic strategies, the dynamic relationship between CsA-mediated modulation of CD26/DPP IV activity and mobilization of EPCs provides a novel target to improve outcomes in patients with ischemia of the myocardium, peripheral limbs, or cerebrum.

**REFERENCES**