Cyclosporin A Enhances Interleukin-8 Expression by Inducing Activator Protein-1 in Human Aortic Smooth Muscle Cells

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Objective—Cyclosporin A (CsA) and tacrolimus (FK506) are widely used as immunosuppressants. However, their use has been hampered by various adverse effects, such as acceleration of atherosclerosis. Interleukin (IL)-8, a chemotactic cytokine, plays an important role in pathogenesis of atherosclerosis. We thus investigated whether synthesis of IL-8 from primary human aortic smooth muscle cells is influenced by CsA and FK506.

Methods and Results—Northern blot analysis revealed that CsA increased IL-8 mRNA level and enhanced its increase by epidermal growth factor or tumor necrosis factor-α. In contrast, FK506 had no effect on the mRNA level. IL-8 accumulation in culture media was also increased by CsA. Stability of IL-8 mRNA was not affected by CsA, whereas luciferase reporter gene assay using the human IL-8 promoter revealed that CsA significantly augmented the promoter activity. Electrophoretic mobility shift assay showed that binding activity of activator protein (AP)-1 was increased by CsA, and introduction of a mutation into the AP-1 site in the promoter abolished its CsA-dependent activation. The increased AP-1 binding activity was accompanied by c-Fos synthesis.

Conclusions—CsA stimulates synthesis of IL-8 via activation of AP-1 in human aortic smooth muscle cells, providing a novel aspect of biological effects of CsA on the cells. (Arterioscler Thromb Vasc Biol. 2003;23:2034-2040.)

Key Words: Cyclosporin A ■ tacrolimus ■ interleukin-8 ■ activator protein-1 ■ human aortic smooth muscle cells
present study, we therefore investigated whether synthesis of IL-8 from human aortic SMCs is influenced by CsA treatment. Our results show that, in contrast to most previous reports, CsA but not FK506 stimulates synthesis of IL-8 via activation of AP-1. It will be also shown that CsA enhances EGF-dependent and TNF-α-dependent synthesis of IL-8.

**Methods**

**Cell Culture**

Primary human aortic smooth muscle cells (HASMCs; Kurabo Industries Limited) were cultured in a growth medium, HuMedia-SB2 medium (Kurabo) supplemented with 5% FBS, 0.5 ng/mL human EGF, 2 ng/mL human fibroblast growth factor (FGF)-B, 5 μg/mL insulin, 50 μg/mL gentamicin, and 50 ng/mL amphotericin B. Nearly confluent HASMCs were then cultured for 4 days in a differentiation medium consisting of HuMedia-SB2 medium supplemented with 1% FBS, 30 μg/mL heparin, 50 μg/mL gentamicin, and 50 ng/mL amphotericin B. Note that the differentiation medium does not contain EGF nor FGF-B. The cells were treated with 2 μmol/L CsA or 2 μmol/L FK506 for 30 minutes, followed by incubation with 30 ng/mL human EGF or 40 ng/mL human TNF-α. After 3-hour incubation, the cells were harvested for electrophoretic mobility shift assay (EMSA) and Western blot analysis after 6 hours for Northern blot analysis. CsA and FK506 were generous gifts from Novartis Pharma and Fujisawa Pharmaceutical, respectively. In an experiment using cycloheximide (CHX), it was added to the media at a concentration of 10 μg/mL 15 minutes before CsA treatment. Jurkat T cells were also used to study the effect of CsA. They were treated with 2 μmol/L CsA for 30 minutes, followed by 1 μmol/L calcium ionophore (A23187) and 30 nmol/L phorbol myristate acetate (PMA) for 6 hours. The cells were then harvested for Northern blot analysis. To analyze stability of IL-8 mRNA, the cells were stimulated with 30 ng/mL EGF for 6 hours. Then a RNA synthesis inhibitor, actinomycin D (ActD), was added to the media at a final concentration of 1 μg/mL. After 1 hour of incubation, the cells were divided into two groups. One was cultured in the presence of 2 μmol/L CsA, and the other in its absence. The cells were harvested for Northern blot analysis at 0, 1, 2, 4, and 6 hours after addition of CsA. The effect of 1 μmol/L A23187 and 30 nmol/L PMA on IL-8 mRNA stability was also studied in the same protocol.

**Northern Blot Analysis**

Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method. Northern blotting and hybridization were performed as previously described. Briefly, 15 μg of total RNA was denatured, electrophoresed, and blotted onto a nylon membrane (Gene Screen Plus; New England Nuclear). Hybridization RNA was denatured, electrophoresed, and blotted onto a nylon membrane (Gene Screen Plus; New England Nuclear). Hybridization was performed using ready-to-use tablet (Roche). The color development was carried out using ready-to-use tablet (Roche).

**Western Blot Analysis**

The cells were washed with PBS and harvested with 50 μL of preheated (85°C) 1× SDS gel loading buffer. After boiling, sonication, and centrifugation, the supernatants were used for Western blot analysis. Samples (100 μg of protein) were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia). After blocking with 5% skimmed milk, the membrane was incubated with goat anti-c-Fos and c-Jun antibodies (Santa Cruz). After wash, the membrane was incubated with alkaline phosphatase–conjugated rabbit anti-goat IgG (Zymed). The color development was carried out using ready-to-use tablet (Roche).

**Measurement of IL-8 in the Media by ELISA**

The cells were grown to near confluence in a 60-mm dish and cultured in the differentiation medium for 4 days. In 2 mL of fresh medium, the cells were treated for 12 hours with 2 μmol/L CsA, 30 ng/mL EGF, and 100 U/mL TNF-α alone or in combination. Then IL-8 concentration in culture media was measured using an ELISA kit (human IL-8 AN’ALYZA Immunoassay Kit; Genzyme Techne).

**Luciferase Reporter Gene Assay**

A DNA fragment of the minimum promoter of the human IL-8 gene (DDBJ accession No. M28130) from the nucleotide positions −133 to +46 was amplified by polymerase chain reaction (PCR) and cloned into NheI/HindIII sites of the promotorless luciferase reporter vector pGL3-Basic (Promega). The plasmid was named p-133. Site-directed mutagenesis of the AP-1 binding site (−126 to −120) was performed by PCR using the following primer: 5′-GCTTGGCGTGGAAGATCTAGTTGCTGCCCATGAGGG-3′ and 5′-CGTCTAGCTGAAAGATCTAGTTGCTGCCCATGAGGG-3′. The first 6 nucleotides of GCTAGC are Nhel site. The AP-1 site, TGACTCA, was mutated to AGATCTA (underlined). This plasmid was named p-133-mutAP1. HASMCs (5×103 cells per well) were seeded in 6-well plates and cultured in the growth medium for 2 days. The cells were then transfected with p-133 or p-133-mutAP1 (300 ng/well) and SV40 promoter-driven β-galactosidase plasmid (100 ng/well, pSV-β-Galactosidase Control Vector, Promega) using Effectene transfection reagent (Qiagen). After transfection, the cells were cultured in the growth medium for 6 hours, and then the medium was replaced with the differentiation medium. After 24 hours, the cells were treated with 2 μmol/L CsA or 30 ng/mL EGF for another 24 hours. Then the cells were harvested and the luciferase and β-galactosidase activities were measured as described in our previous report. The luciferase activity was normalized by β-galactosidase activity.

**Reverse Transcriptase–PCR**

Procedures for reverse transcriptase (RT)-PCR were described in our previous reports. The primers for CXCR-1 and CXCR-2 were designed according to Hayes et al, as follows: CXCR-1: 5′-GGGGCCACACAAACCTTC-3′ and 5′-AGTGGCTGCC-TCAATGTCCTC-3′; CXCR-2: 5′-CCGGCGCTGGTGTGAG-3′ and 5′-CTGCCCTTTGTGGCTGTAATA-3′.

**DNA Synthesis**

DNA synthesis in HASMCs was assessed by measurement of incorporation of H-thymidine into the cells. HASMCs were grown in 12-well culture plates to 70% confluence and then made quiescent with differentiation medium for 4 days. The cells were then treated with 2 μmol/L CsA or with 40 or 400 ng/mL of human recombinant IL-8 (Sigma) for 24 hours. ‘H-thymidine (1 μCi per well) was added to the media, followed by an additional 4-hour incubation. Incorporation of ‘H-thymidine was assessed by measurement of the radioactivity in the trichloroacetic acid–insoluble fraction of the cells, as described in our previous report.
requires de novo protein synthesis, the cells were pretreated with 2 μmol/L CsA for 30 minutes followed by 30 ng/mL EGF or 40 ng/mL TNF-α for 6 hours. Northern blot analysis was performed using IL-8 and GAPDH cDNA as probes. Representative images by BAS 2000 system are shown. One experiment was done in duplicate. IL-8 mRNA levels were normalized by those of GAPDH mRNA.

Quantitative results from 2 separate experiments were expressed as mean±SD (n=4). *P<0.05, **P<0.01 vs unstimulated cells. #P<0.01.

Statistical Analysis

Statistical analysis was carried out using 1-way fractional ANOVA, followed by Fisher’s protected least-significant difference analysis.

Results

CsA But Not FK506 Increases IL-8 mRNA Levels

Primary HASMCs cultured in the differentiation medium for 4 days were treated with 2 μmol/L CsA (A) or 2 μmol/L FK506 (B) for 30 minutes followed by 30 ng/mL EGF or 40 ng/mL TNF-α for 6 hours. Northern blot analysis was performed using IL-8 and GAPDH cDNA as probes. Representative images by BAS 2000 system are shown. One experiment was done in duplicate. IL-8 mRNA levels were normalized by those of GAPDH mRNA.

Quantitative results from 2 separate experiments were expressed as mean±SD (n=4). *P<0.05, **P<0.01 vs unstimulated cells. #P<0.01.

Figure 1. CsA but not FK506 increases IL-8 mRNA levels. HASMCs maintained in the differentiation medium for 4 days were treated with 2 μmol/L CsA (A) or 2 μmol/L FK506 (B) for 30 minutes followed by 30 ng/mL EGF or 40 ng/mL TNF-α for 6 hours. Northern blot analysis was performed using IL-8 and GAPDH cDNA as probes. Representative images by BAS 2000 system are shown. One experiment was done in duplicate. IL-8 mRNA levels were normalized by those of GAPDH mRNA.

CsA Does Not Affect IL-8 mRNA Stability

Concentrations of IL-8 in culture media produced from HASMCs were determined by ELISA. As shown in Figure 2, treatment of the cells with CsA for 12 hours resulted in a significant increase in IL-8 accumulation in culture media. Treatment with EGF or TNF-α also significantly increased IL-8 accumulation, and CsA additionally increased the levels. The IL-8 concentrations in culture media well reflect its mRNA levels shown in Figure 1A.

Figure 2. CsA stimulates synthesis of IL-8 protein. The differentiated HASMCs in a 60-mm dish were treated with 2 μmol/L CsA for 30 minutes followed by 30 ng/mL EGF or 40 ng/mL TNF-α for 12 hours. IL-8 concentrations in the culture media (2 mL) were determined by ELISA. The experiment was done in triplicate. Values were expressed as mean±SD. *P<0.01 vs unstimulated cells. #P<0.01.

CsA Stimulates Synthesis of IL-8

Concentrations of IL-8 in culture media produced from HASMCs were determined by ELISA. As shown in Figure 2, treatment of the cells with CsA for 12 hours resulted in a significant increase in IL-8 accumulation in culture media. Treatment with EGF or TNF-α also significantly increased IL-8 accumulation, and CsA additionally increased the levels. The IL-8 concentrations in culture media well reflect its mRNA levels shown in Figure 1A.

Expression of human IL-8 gene is regulated at the transcriptional and posttranscriptional levels.

We first evaluated effects of CsA on IL-8 mRNA stability, as shown in Figure 3. Expression of IL-8 mRNA was induced by EGF. Then a RNA synthesis inhibitor ActD was added and the cells were cultured in the presence or absence of CsA. Northern blot analysis revealed the long half-life of IL-8 mRNA in HASMCs; ~75% of mRNA was detected after 6 hours of incubation with ActD in the absence of CsA. Similar levels of the mRNA were detected in the presence of CsA, demonstrating that CsA does not increase IL-8 mRNA stability. It was also found that stability of the mRNA induced by calcium ionophore and PMA was similar to that induced by EGF and that CsA had no effect on the stability (data not shown).

CsA Enhances DNA-Binding Activity of AP-1

It has been shown that the human IL-8 promoter sequence spanning nucleotides −133 to −1 containing AP-1,
and C/EBPβ binding sites is essential and sufficient for transcriptional regulation of the gene. We thus examined effect of CsA on DNA-binding activities of these 3 transcription factors by EMSA. Binding activity to the AP-1 site (−126 to −120) in human IL-8 promoter was shown in Figure 4A. Treatment of the cells with CsA substantially increased the AP-1 binding activity (lane 2) above the basal level (lane 1). EGF also increased the binding activity (lane 3), and CsA additionally increased the activity (lane 4). Although TNF-α alone had only marginal effect on AP-1 binding (lane 5), TNF-α together with CsA augmented the binding activity (lane 6). When supershift analysis was performed using the extracts obtained from the cells treated with EGF and CsA, the AP-1 band was supershifted by anti-c-Fos antibody (lane 7) and its density was markedly reduced by anti-c-Jun antibody (lane 8) compared with the density of lane 4. Anti-Fra1, anti-Fra2, and anti-FosB antibodies had no effect on the AP-1 binding (data not shown).

Similar results were obtained when canonical AP-1 oligonucleotide derived from human collagenase promoter was used as a probe (data not shown). These results indicate that the binding activity of AP-1 consisting of cFos/c-Jun heterodimer is increased by CsA.

Binding activity to the NF-κB site (−81 to −72) was shown in Figure 4B. Two retarded bands were evident in all lanes. The lower band with the asterisk represents nonspecific binding. When HASMCs were treated with TNF-α, the density of the upper band increased (lane 5) above the basal level (lane 1). This band was supershifted by anti-p56 antibody (lane 7) but not by anti-p50 (lane 8), p52, and c-Rel antibody (data not shown) antibodies, suggesting that this NF-κB consists of p65 homodimer. The result is consistent with the previous report. On the other hand, EGF did not activate NF-κB (lane 3). In contrast to the AP-1 binding, CsA did not exchange NF-κB binding in the basal condition (lane 2) nor in the cells treated with EGF (lane 4) and TNF-α (lane 6).

The binding activity to the C/EBPβ site (−93 to −84) was also investigated by EMSA (data not shown). A single band was detected in unstimulated cells, and its density was markedly reduced by anti-C/EBPβ antibody. The density of the band was not altered by CsA, EGF, and TNF-α, indicating that C/EBPβ binding is not altered by treatment with CsA.

Taken together, these results suggest that TNF-α stimulates expression of IL-8 gene by activating NF-κB, whereas EGF does by increasing AP-1. Under both conditions as well as in unstimulated cells, CsA increases AP-1 binding to IL-8 promoter.

**Mutation of the AP-1 Site in IL-8 Promoter Inhibits CsA-Dependent Promoter Activation**

To confirm the role of AP-1 in CsA-dependent IL-8 gene expression, the plasmids containing a luciferase reporter gene driven by the minimum IL-8 promoter (p-133) or its mutant for AP-1 site (p-133-mutAP1) were constructed and a reporter gene assay was performed in HASMCs. As shown in Figure 5A, when p-133 was transfected, its basal activity was detected, and treatment of the transfected cells with CsA significantly increased the promoter activity by 4-fold. EGF also significantly increased the activity, and CsA increased it additionally. These results correlate well with those of Northern blot analysis (Figure 1A). In contrast, when p-133-mutAP1 was transfected, the stimulatory effects of CsA and EGF were abolished. These data indicate that the increased AP-1 binding to the AP-1 site located in −126 to −120 in human IL-8 promoter is responsible for CsA-dependent expression of IL-8 gene in HASMCs.
CsA Increases c-Fos Protein Synthesis

Western blot analysis was performed to evaluate effect of CsA on c-Fos and c-Jun protein levels in HASMCs. As shown in Figure 5B, a single band of \( \text{H}11015 \text{60 kDa} \) was detected by anti-c-Fos antibody. The size of this band was compatible with that of the human c-Fos protein and different from those of other Fos family members such as Fra-1, Fra-2, and FosB. Treatment of the cells with CsA increased the c-Fos protein level. Treatment with EGF also increased the level, and combined treatment with CsA resulted in additional increase. Anti-c-Jun antibody detected a single band of \( \text{H}11015 \text{40 kDa} \) protein. In contrast to c-Fos, c-Jun protein levels were not altered by CsA nor EGF. These results indicate that the AP-1 binding activities increased by CsA and EGF are attributable, at least in part, to the augmented c-Fos synthesis.

IL-8 Stimulates DNA Synthesis in HASMCs

IL-8 belongs to CXC chemokine family, and its action is exerted through the membrane receptors CXCR1 and CXCR2. Thus, expression of these receptors was examined in HASMCs. As shown in Figure 6A, when RT products from HASMCs cultured in the growth medium were used, a single band was amplified by RT-PCR using CXCR1 primers and CXCR2 primers. The sizes of the bands were consistent with the expected lengths of the amplified products, 363 bp for CXCR1 and 385 bp for CXCR2, demonstrating expression of these receptors in HASMCs. Interestingly, when the cells were cultured in the differentiation medium, the densities of amplified bands were increased, suggesting that differentiation of HASMCs may stimulate expression of CXCR1 and CXCR2. On the other hand, no bands were amplified from the samples in which RT was omitted from reverse transcription reaction (data not shown), indicating that contamination of our RNA samples with genomic DNA was, if any, below the detectable level.

Effects of CsA and IL-8 on proliferation of HASMCs were then assessed by \( \text{H}^{3} \)-thymidine incorporation into the cells. As shown in Figure 6B, treatment of the cells with 2 \( \mu \text{mol/L} \) CsA or with 40 ng/mL IL-8 did not increase thymidine incorporation. However, 400 ng/mL IL-8 significantly stimulated thymidine incorporation of HASMCs.

Discussion

The present study for the first time demonstrates upregulation of IL-8 expression by CsA in HASMCs. Furthermore, EGF-dependent and TNF-\( \alpha \)-dependent IL-8 expression in the cells is also enhanced by CsA. Although several reports have demonstrated inhibitory effect of CsA on IL-8 expression in various cell types, such as T cells, eosinophils, neutrophils, monocytes, keratinocytes, and cardiac myxoma...
to inhibition of calcineurin activity by CsA. However, this mechanism is unlikely in HASMCs, because FK506, another calcineurin inhibitor, failed to increase IL-8 mRNA level in the cells. Additional studies are required to elucidate the mechanisms of CsA action on c-Fos.

Yue et al.11 reported that IL-8 stimulates proliferation of rat and human SMCs. Also, Simonini et al.47 showed that neutralization of IL-8 in culture media by its antibody inhibits growth of human coronary SMCs. However, IL-8 action on SMCs is ambiguous, because some reports indicated that IL-8 binding and its receptor expression were not detected in human SMCs.56,48 In contrast to these observations, it was clearly demonstrated that HASMCs express CXCR1 and CXCR2 and that 400 ng/mL IL-8 increased DNA synthesis. These results indicate the presence of functional receptor for IL-8 in HASMCs. The controversy of expression of CXCR1 and CXCR2 may be attributable to culture conditions for SMCs, because our result indicated that the presence of 5% FBS, EGF, and FGF-B in culture media tended to reduce the expression of CXCR1 and CXCR2. On the other hand, CsA had no effect on DNA synthesis. This may be attributable to the low level of IL-8 production by CsA or antiproliferative effects of CsA on HASMCs.

The IL-8 concentration required for the increase in DNA synthesis is 400 ng/mL, which is much higher than the concentration in culture medium observed after the combined treatment with CsA and TNF-α for 12 hours (44 ng/mL in Figure 2). However, it is possible that CsA-dependent IL-8 production in the microenvironment in vivo could result in the local concentration much higher than that in cell culture system. Additional studies are thus required to confirm the stimulatory effect of CsA on proliferation of HASMCs.

In conclusion, the present study demonstrates that CsA increases IL-8 gene expression by stimulating AP-1 DNA binding after c-Fos synthesis in HASMCs. These findings provide a novel aspect of biological effects of CsA on the cells. In addition, considering the role of IL-8 in development of atherosclerosis,7,9,10 the enhanced IL-8 expression by CsA in HASMCs could be one of the mechanisms for acceleration of posttransplant atherosclerosis.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan. The authors are indebted to Dr. David Zadworny (McGill University, Canada) for his critical reading of the manuscript.

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Arterioscler Thromb Vasc Biol. 2003;23:2034-2040; originally published online September 4, 2003;
doi: 10.1161/01.ATV.000094234.60166.78

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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