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Cyclosporin-A inhibits the expression of cyclooxygenase-2 in gingiva

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Background and Objective: Various inflammatory mediators are involved in the development of cyclosporine A-induced gingival overgrowth. In this study, the gingival expression of cyclooxygenase-2 after cyclosporine A therapy was examined *in vivo* and *in vitro*.

Material and Methods: After edentulous ridges on maxilla were established, 21 Sprague-Dawley rats received cyclosporine A daily for 4 wk, and a further 21 rats received solvent. After the rats were killed, the expression of cyclooxygenase-2 mRNA, interleukin-1β mRNA, tumor necrosis factor-α mRNA, and interleukin-6 mRNA was examined in the edentulous gingiva. The expression of cyclooxygenase-2 protein and the production of prostaglandin E_2 were also evaluated.

Results: In cultured human gingival fibroblasts and epithelial cells, the expression of cyclooxygenase-2 mRNA was measured after treatment with cyclosporine A. Significantly lower expression of cyclooxygenase-2 and interleukin-1β mRNA, but higher interleukin-6 expression, were observed in gingiva from cyclosporine A-treated rats than in those from the control rats. Significantly less prostaglandin E_2 production was observed in cyclosporine A-treated rats. Immunohistochemistry revealed that fewer gingival stromal cells were positively stained for cyclooxygenase-2 in cyclosporine A-treated rats. In cultured cells, significantly less cyclooxygenase-2 mRNA was detected after treatment with cyclosporine A.

Conclusion: The expression of cyclooxygenase-2 was lower in the plaque nonretentive gingivae and the *in vitro* gingival cells upon treatment with cyclosporine A. Thus, we propose that cyclosporine A inhibits the expression of gingival cyclooxygenase-2.

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Cyclosporin A-induced gingival overgrowth, a side-effect of cyclosporine A therapy, is characterized by epithelial hyperplasia, interstitial fibrosis, and focal inflammatory cell infiltration (1–3). Although it has been suggested that local factors, such as dental bacterial plaque, are significantly associated with this overgrowth (4), periodontal destruction and attachment loss does not seem to be the major pathological finding. In animals, overgrowth is exacerbated when dental plaque is retained, but a reduction in overgrowth has been observed when chlorhexidine is applied (5,6). In humans, adequate plaque control improves the condition of the gingiva, but does not totally prevent the development of overgrowth (7). Complex interactions among the various inflammatory mediators and tissue modeling may be involved in the pathogenic mechanisms of overgrowth.

A variety of cytokines and growth factors that mediate the host response are produced by the cells of periodontal tissues (8). Recently, the roles, in cyclosporine A-induced gingival overgrowth, of various growth factors, including transforming growth factor-β, platelet-derived growth factor, and vascular endothelial growth factor (9,10), and of pro-inflammatory cytokines, such as interleukin-1β, tumor necrosis factor-α, and interleukin-6

(11–13), have been examined. Cyclooxygenase-2 expression is significantly up-regulated in inflamed periodontal tissues (14). However, the role of cyclooxygenase-2 in the development of cyclosporine A-induced overgrowth has never been evaluated. In this study, the expression of cyclooxygenase-2 and the production of its end product, prostaglandin E_2, in the gingiva after cyclosporine A treatment, without the influence of bacterial plaque, were investigated *in vivo* and *in vitro* to elucidate the role of cyclooxygenase-2 in the induced gingival overgrowth.

Material and methods

Experimental design

In vivo — Edentulous ridges were established in 42, 5-wk-old male Sprague-Dawley rats (15). Briefly, all right maxillary molars were extracted under general anesthesia induced with chloral hydrate (Ferak Berlin GmbH, Berlin, Germany). After a 3-wk healing period, the rats were randomly assigned to either the cyclosporine A group or the control group (21 rats in each group). Animals in the cyclosporine A group received 30 mg/kg of cyclosporine A (Sandimmun; Sandoz, Basel, Switzerland), daily by gastric feeding for 4 wk, and the control rats received only mineral oil. At the end of the study, the animals were killed by carbon dioxide inhalation and the edentulous tissues were dissected. Sixteen specimens collected from each group were immediately frozen in liquid nitrogen and stored at −70°C until use. Five specimens from each group were fixed in 4% paraformaldehyde for histological examination.

In vitro — A healthy gingival specimen was obtained from a distal wedge during a flap operation on the upper palatal region of a 40-year-old female undergoing periodontal surgery at the Dental Clinic of the Tri-Service General Hospital, Taipei, Taiwan. After the specimen was immersed in Leibovitz L-15 medium (Sigma-Aldrich Inc., St Louis, MO, USA), supplemented with 2 mg/mL of dispase II (Roche Diagnostics, Indianapolis, IN, USA) and 10% fetal bovine serum, at 4°C for 2 d, the epithelial layer was separated from the underlying connective tissue. Both layers were minced into small pieces. The epithelial fragments were digested in serum-free medium containing 0.05% trypsin–EDTA (Gibco BRL, Life Technologies, Grand Island, NY, USA) at 37°C in 5% CO_2 for 5 min. The connective tissue fragments were digested in medium containing 10% fetal bovine serum and 2 mg/mL of collagenase (Sigma-Aldrich Inc.) for 24 h. The fragments were placed in culture flasks to allow the cells to migrate from the explants. The epithelial cell cultures were maintained in EpiLife™ medium supplemented with 1% human keratinocyte growth supplement (Cascade Biologics™, Portland, OR, USA), and incubated until a monolayer formed. The fibroblast cultures were maintained in 10% fetal bovine serum in Dulbecco's modified Eagle's minimal essential medium/F-12. The cells were incubated until they reached 70% confluence and the medium was then replaced with new medium (1% fetal bovine serum for fibroblasts and growth-factor-free medium for epithelial cells). After stimulation with a final concentration of 100 ng/mL of cyclosporine A (16) in ethanol for 24 h, the cells were harvested to evaluate the expression of cyclooxygenase-2 mRNA by reverse transcription-polymerase chain reaction (RT-PCR).

RNA extraction and RT-PCR

The samples were homogenized in Trizol reagent (Invitrogen, Grand Island, NY, USA) with a glass homogenizer, and the total RNA was quantified by spectrophotometry at 260 nm. cDNA was generated with SuperScript III using the GeneAmp®-9700 PCR System (Applied Biosystems, Foster City, CA, USA). The polymerase chain reaction (PCR) was carried out for 30 to 40 cycles to allow quantitative comparison of the cDNAs obtained. Reactions involved denaturation at 94°C, annealing at 58–64°C, and extension at 72°C (10). The PCR primers used in the animal study were as follows: cyclooxygenase-2 primers (17), 5′-TTTGTTGAGTCATTCACCAGACAGAT-3′ and 5′-CAGTATTGAGGAGAACAGATGGGATT-3′; interleukin-1 primers (18), 5′-GACCTGTTCTTTGAGGCTGAC-3′ and 5′-TTCATCTCGAAGCCTGCAGTG-3′; tumor necrosis factor-α primers (19), 5′-AGTCTTCCAGCTGGAGAAGG-3′ and 5′-GCCACTACTTCAGCATCTCG-3′; interleukin-6 primers (20), 5′-GACTGATGTTGTTGACAGCCACTGC-3′ and 5′-TAGCCACTCCTTCTGTGACTCTAACT-3′; and glyceraldehyde-3-phosphate dehydrogenase primers (21), 5′-TGCTGGTGCTGAGTATGTCG-3′ and 5′-TGCTGGTGCTGAGTATGTCG-3′. The primers used in the cultures of human gingival cells were: cyclooxygenase-2 primers, 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ and 5′-AGATCATCTCTGCCTGAGTATCTT-3′; and glyceraldehyde-3-phosphate dehydrogenase primers, 5′-AGCCGCATCTTCTTTTGCGTC-3′ and 5′-TCATATTTGGCAGGTTTTTCT-3′ (22,23). The amplified products were analysed on agarose gels stained with ethidium bromide. RT-PCR products were scanned and normalized relative to the glyceraldehyde-3-phosphate dehydrogenase bands.

Western blotting for cyclooxygenase-2 and interleukin-1β in the gingival tissues of rats

The gingival homogenates from two animals were pooled, lysed, and boiled. Protein concentrations were determined using the BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Tissue extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on polyacrylamide gels and then electroblotted onto poly(vinylidene difluoride) membrane. After blocking with 5% fetal bovine serum for 1 h, the membranes were hybridized with polyclonal anti-cyclooxygenase-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or with antibody to interleukin-1β (Abcam, Cambridge, UK). After the samples were washed with phosphate-buffered saline, they were incubated with horseradish peroxidase-conju-

gated secondary antibodies and detected with enhanced chemiluminescence.

Extraction and enzyme immunoassay for prostaglandin E_2

Gingival tissues were homogenized in 1.5 mL of phosphate-buffered saline with a glass homogenizer. Prostaglandin E_2 was extracted from the homogenate with a solid-phase extraction method using a cartridge (24). The gingival extracts were diluted with an ethanol–water mixture (1 : 4) and passed through columns that had been prewashed with absolute ethanol, at a slow flow rate. The columns were washed twice with 20% ethanol and then with 3 mL of hexane solution. Prostaglandin E_2 was eluted with 5 mL of ethyl acetate solution, and dried in an evaporator (N-EVAP; Organomation Associates, Inc., South Berlin, MA, USA) under a gentle stream of nitrogen. Prostaglandin E_2 was quantified using an enzyme immunoassay system (Prostaglandin E_2 Biotrak Enzymeimmunoassay System; Amersham Biosciences, Bucks., UK). Optical density was read by using an enzyme-linked immunosorbent assay plate reader (Dynex Technologies Inc., Chantilly, VA, USA) at a wavelength of 450 nm.

Immunohistochemical staining

Serial cryostat sections of the edentulous gingivae were sliced buccopalatally to a thickness of 5 μm. The sections were incubated with 0.1% hydrogen peroxide for 30 min to quench their endogenous peroxidase activity. After they had been washed, the sections were incubated for 4 h with unconjugated primary polyclonal antibody directed against cyclooxygenase-2. The sections were then incubated with biotinylated secondary antibody for 30 min, followed by incubation with streptavidin-conjugated horseradish peroxidase complexes for 30 min and in 3-amino-9-ethyl carbazole solution for 20 min. The sections were counterstained with hematoxylin. The percentages of cells staining positive for cyclooxygenase-2 were recorded after counting the total gingival stromal cells in 0.0324 mm^2 of gingival connective tissue, as in our previous study (10).

Statistical analysis

The Student's *t*-test was used to evaluate the differences between the cyclosporine A and control groups in the expression of cytokine mRNAs and proteins, and of prostaglandin E_2 concentrations. A *p*-value of < 0.05 was deemed to be significant.

Results

The expression of cyclooxygenase-2 and interleukin-1β mRNAs was significantly weaker in gingival tissues from the cyclosporine A group than in those from the controls, whereas the expression of interleukin-6 mRNA was significantly stronger in gingival tissues from the cyclosporine A group (Fig. 1). No significant difference in tumor necrosis factor-α expression was noted between the two groups.

Western blotting indicated that the expression of cyclooxygenase-2 and interleukin-1β proteins was significantly weaker in the gingivae of the cyclosporine A group than in that of the control group (Fig. 2, left). Enzyme immunosorbent assay results revealed that prostaglandin E_2 production was significantly lower in the gingivae of the cyclosporine A group than in the gingivae of the control group (Fig. 2, right). Immunohistochemical staining revealed that the gingival stromal cells, including fibroblasts and endothelial

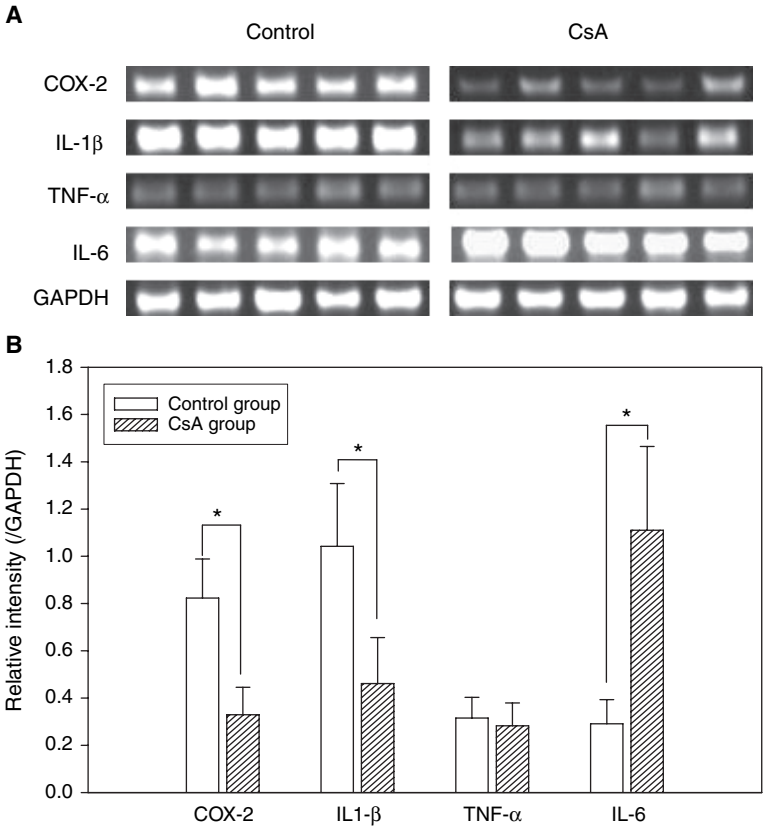

Fig. 1. The expression of mRNA for cyclooxygenase-2, interleukin-1β, tumor necrosis factor-α, and interleukin-6 in the edentulous gingivae of control and cyclosporine A-treated rats. The gel images of reverse transcription-polymerase chain reaction products, amplified from cyclooxygenase-2 and cytokine mRNAs in gingival tissues from five control and five cyclosporine A-treated rats, are shown (A). Comparison of the relative intensities of cyclooxygenase-2 and cytokine mRNAs in the gingivae of the control and cyclosporine A groups (B). Data are expressed as means and standard deviations. *Significantly different at $p < 0.001$. COX-2, cyclooxygenase-2; CsA, cyclosporine A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; TNF-α, tumor necrosis factor-α.

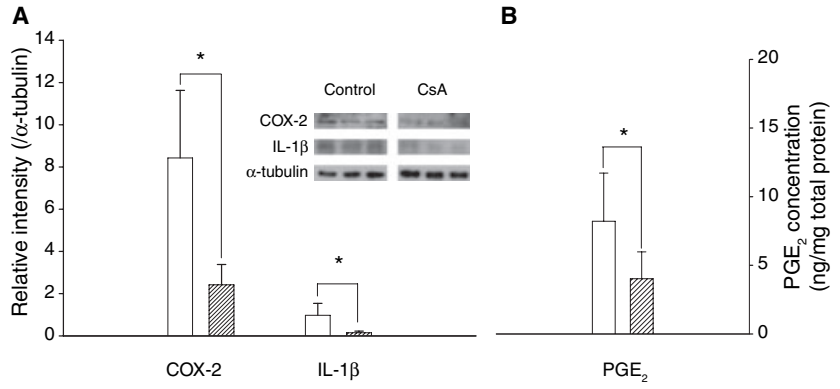

Fig. 2. Comparison of rat edentulous gingivae between the control group (white bar) and the cyclosporine A-treated group (striped bar) in the expression of cyclooxygenase-2 and interleukin-1β proteins (A), and in the production of prostaglandin E_2 (B). Data are expressed as mean values and standard deviations. *Significantly different at $p < 0.05$. Each band of cyclooxygenase-2, interleukin-1β, and α-tubulin proteins for western blot, shown in the insert, were pooled from two rats. COX-2, cyclooxygenase-2; CsA, cyclosporine A; IL, interleukin; PGE_2, prostaglandin E_2.

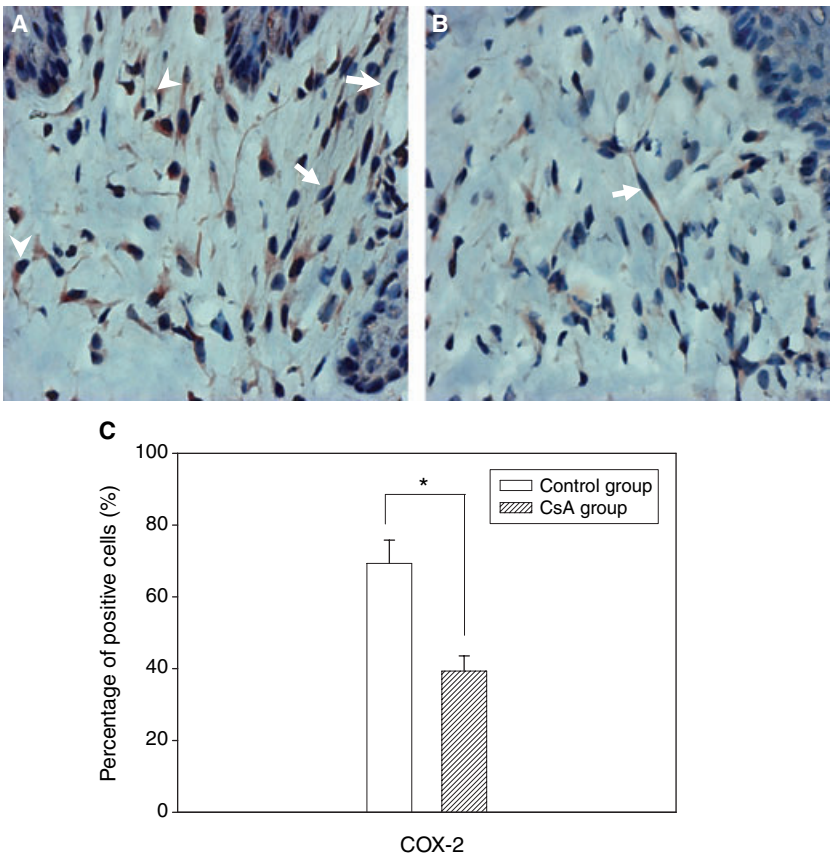

Fig. 3. Micrographs showing immunohistochemical staining for cyclooxygenase-2 in gingival tissues from a control rat (A) and from a cyclosporine A-treated rat (B). Positively stained stromal cells, including fibroblasts (arrows) and endothelial cells (arrowhead), were observed (original magnification, ×100). Percentages of cells staining positively for cyclooxygenase-2 were compared in the gingival tissues between control and cyclosporine A groups by histometry (C). COX-2, cyclooxygenase-2; CsA, cyclosporine A.

cells, were positively stained for cyclooxygenase-2 in the control and cyclosporine A groups (Fig. 3A,B). A significantly higher percentage of cells positively stained for cyclooxygenase-2 was noted in the gingiva of the control group than in those of the cyclosporine A group, by histometric measurement (Fig. 3C).

The expression of cyclooxygenase-2 mRNA was significantly lower in cultured human gingival fibroblasts and epithelial cells after cyclosporine A treatment than in those without treatment (Fig. 4).

Discussion

In this study, rats of the cyclosporine A treatment group exhibited weaker expression of cyclooxygenase-2 and prostaglandin E_2 in their edentulous gingival tissue than did rats of the control group. The expression of cyclooxygenase-2 mRNA was also reduced in primary cultures of gingival cells after cyclosporine A treatment. In a murine macrophage cell line, cyclosporine A was shown to inhibit cyclooxygenase-2 expression at the mRNA level, leading to significant inhibition of prostaglandin E_2 production. The authors therefore attributed potential anti-inflammatory properties to cyclosporine A, in addition to its known immunosuppressive activity (25). Recently, a study showed that cyclosporine A inhibited the up-regulation of cyclooxygenase-2 mRNA in peripheral blood lymphocytes activated with phytohemagglutinin E and phorbol ester (26).

Prostaglandin E_2 production and cyclooxygenase-2 expression may be associated with the destruction of connective tissue and alveolar bone resorption in the periodontal tissue (27). After the topical application of lipopolysaccharide from *Escherichia coli* (5 mg/mL) to the rat molar gingival sulcus, consequent transient expression of cyclooxygenase-2 was observed in the junctional epithelial cells, whereas no up-regulation of cyclooxygenase-1 expression was caused by this application. Lipopolysaccharide application also caused a transient up-regulation of cyclooxyge-

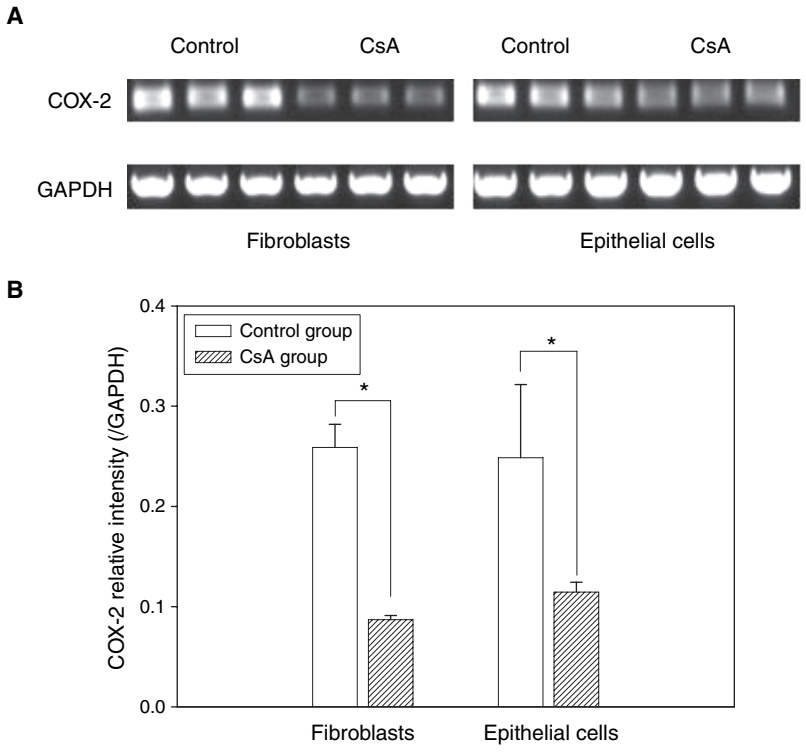

Fig. 4. The expression of cyclooxygenase-2 mRNA in cultured human gingival fibroblasts and epithelial cells after cyclosporine A treatment. Gel images of cyclooxygenase-2 mRNA (A) and a comparison of their relative intensities (B) in cultured cells, with and without cyclosporine A treatment. Data are expressed as mean values and standard deviations. *Significantly different at $p < 0.05$. COX-2, cyclooxygenase-2; CsA, cyclosporine A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

nase-2 expression in periodontal ligament fibroblasts, cementoblasts, and osteoblasts (27). Although the exact role of cyclosporine A in the tissue destruction of periodontal disease is still unknown, the effect of cyclosporine A on prostaglandin E_2 production in human gingival fibroblasts challenged with tumor necrosis factor-α has been studied. Tumor necrosis factor-α stimulated prostaglandin E_2 production dose-dependently in 24-h cultures. However, cyclosporine A alone did not directly induce prostaglandin E_2 formation but potentiated tumor necrosis factor-α to induce prostaglandin E_2 formation in gingival fibroblasts in a manner dependent on the concentrations of both cyclosporine A and tumor necrosis factor-α (28). Another study showed that cyclosporine A did not inhibit cyclooxygenase-2 expression in gingival fibroblasts challenged with *Actinobacillus actinomycetemcomitans* or *Bacterioides forsythus* (14). In the same study, cyclooxygenase-2 expression was significantly higher in inflamed human periodontal tissues *in vivo* and in cultured human gingival fibroblasts challenged with interleukin-1β or bacterial pathogens *in vitro*. Thus, a suitable model, avoiding the influences of periodontal plaque, is required for elucidating the detailed roles of various inflammatory mediators in the pathogenic mechanisms of overgrowth. In the present study, the expression of gingival cyclooxygenase-2 upon cyclosporine A treatment was examined in both animal experiments of plaque nonretentive gingivae and *in vitro*-cultured human gingival cells.

Because cyclosporine A administration induces nephrotoxicity, the production of prostaglandin E_2 and the expression of cyclooxygenase-1 and cyclooxygenase-2 mRNAs have been evaluated in cultured mouse renal ascending limb epithelial cells, with or without cyclosporine A (16). The cells produced prostaglandin E_2, mainly via cyclooxygenase-1 in the steady state and via cyclooxygenase-2 in the lipopolysaccharide-stimulated state. Cyclosporine A dose-dependently reduced the production of prostaglandin E_2 in renal epithelial cells in the lipopolysaccharide-stimulated state. The inhibition of cyclooxygenase-2 expression, which may in part account for the adverse effects of cyclosporine A in the kidney, was further examined in animal studies (29,30). The involvement of cyclooxygenase-derived prostaglandins in vascular endothelial function outside the transplanted organ was also strongly diminished after allogeneic renal transplantation (31).

Cyclosporine A inhibits the migration of primary endothelial cells and the angiogenesis induced by vascular endothelial growth factor. This effect appears to be mediated through the inhibition of cyclooxygenase-2 (32). The cyclosporine A-mediated inhibition of angiogenesis was attributable to the cyclooxygenase-2 inhibitor both *in vitro* and *in vivo*, and was reversed by prostaglandin E_2. Moreover, the induction of cyclooxygenase-2 gene expression by vascular endothelial growth factor requires the activation of the nuclear factor of activated T cells (32). In primary cultures of human intestinal microvascular endothelial cells, further activation of p44/42 mitogen-activated protein kinase and partial inhibition of Jun N-terminal kinase and p38 mitogen-activated protein kinase are involved in cyclosporine A-blocked vascular endothelial growth factor-induced angiogenesis (33). Cyclosporine A inhibited endothelin-1-induced cyclooxygenase-2 expression via the nuclear factor of activated T-cell transcription factor in cultured rat glomerular mesangial cells (34). The impairment of dendritic cell migration by the inhibition of prostaglandin E_2 production and the regulation of chemokine receptor expression may contribute to cyclosporine A-mediated immunosuppression (35). Recently, we demonstrated, in our laboratory, the increased expression of vascular endothelial growth factor (10) and

endothelin-1 in the gingivae of rats receiving cyclosporine A therapy (unpublished data). However, the exact pathogenesis or disease pathway of cyclosporine A-induced gingival changes is still unknown.

The expression of interleukin-1β and tumor necrosis factor-α was evaluated in the present study because of their potent induction of cyclooxygenase-2 expression and prostaglandin E_2 production (28,36). Cyclosporine A inhibits interleukin-1β and tumor necrosis factor-α production in cultured gingival fibroblasts (37). Cyclosporine A also increases the interleukin-6 secretion from gingival fibroblasts derived from either healthy gingival tissue (11) or overgrown gingiva (12). Increased levels of interleukin-6 protein and interleukin-6 mRNA have been demonstrated in cyclosporine A-induced overgrown human gingival tissues (13). In the present study, the down-regulation of interleukin-1β and the up-regulation of interleukin-6 were observed after cyclosporine A therapy, but no significant effect on tumor necrosis factor-α production was noted (Fig. 1). In conclusion, based on our findings from *in vivo* and *in vitro* experiments, we suggest that cyclosporine A inhibits gingival cyclooxygenase-2 expression, which may partly explain the sign of minimal periodontal destruction and attachment loss in the gingival overgrowth.

Acknowledgements

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