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Expression and bioactivities of endothelin-1 in gingiva during cyclosporine A treatment

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Background and Objective: This study aimed to evaluate the expression and bioactivities of endothelin-1 (ET-1) in gingiva during cyclosporine A (CsA) treatment.

Material and Methods: After establishing edentulous ridges, experimental rats were fed 30 mg/kg/day CsA while control animals received mineral oil for 4 weeks, after which a reverse transcription-polymerase chain reaction (RT-PCR) and/or immunohistochemistry was used to examine the expression of ET-1, its receptors, proliferating cell nuclear antigen (PCNA) and inducible nitric oxide synthase (iNOS) in gingivae. The roles of the endothelin receptors A and B (ET_A and ET_B) in CsA-enhanced expression of PCNA and iNOS were examined in cultured human gingival fibroblasts pretreated with receptor antagonists, by immunocytochemistry and RT-PCR, respectively.

Results: The mRNA expression of ET-1, ET_A and ET_B, as well as of PCNA and iNOS, was significantly greater in edentulous gingiva that received CsA compared with control gingiva. Immunohistochemistry revealed more cells positively stained for ET-1 and its receptors in the tissues of CsA-treated rats than in those of control rats. In fibroblast cultures, enhanced mRNA expression of ET-1, ET_A and ET_B was observed after CsA treatment at the concentrations of 10 and 100 ng/mL. Cyclosporine A-enhanced PCNA expression was somewhat reduced by blockade of ET_A, but not ET_B, whereas iNOS expression was somewhat reduced by blockade of ET_B.

Conclusion: Based on the present findings, we suggest that: (1) CsA upregulates the gingival expression of ET-1 and its receptors; and (2) ET_A and ET_B have different bioactivities, ET_A being involved in cell proliferation and ET_B being associated with iNOS expression.

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Gingival overgrowth is one of the side-effects observed in patients receiving cyclosporine A (CsA), an immunosuppressant. The induced overgrowth presents as an increased gingival volume, including an increased number of cells and a higher level of extracellular matrix production (1,2), but the mechanisms underlying this effect are still under investigation.

Endothelin-1 (ET-1), a 21-amino-acid vasoconstrictor peptide synthesized by a range of normal cell types, displays a range of biological functions (3), including being mitogenic for a number of cell types, such as fibroblasts (4). It has been shown that ET-1 is not only a strong vasoconstrictor but

is also a growth-promoting factor (5). Recent studies have also shown that ET-1 modifies extracellular matrix metabolism in dermal fibroblasts (6,7). A biphasic effect of ET-1 on blood vessels has been observed: it mediates vascular smooth muscle contraction but induces nitric oxide (NO), a vasodilator, in endothelial cells (8). Unfortunately, studies concerning the role of ET-1 in the development of CsA-induced gingival overgrowth are rare. A recent report found that, compared with healthy control subjects, the expression and synthesis of ET-1 and its receptors (endothelin receptor A and B; ET_A and ET_B) were increased in gingival tissue from patients with chronic periodontitis and gingival overgrowth (9). In addition, an increased level of ET-1 was also found in cultured gingival fibroblasts after CsA treatment. The authors concluded that CsA could modulate the expression of ET-1 during CsA-induced gingival overgrowth. However, they also suggested that microbe-induced inflammation, a prerequisite for the induction of gingival overgrowth in their model, might influence the expression of ET-1. Since the expression of ET-1 is positively associated with periodontal inflammation (10), further detailed studies without the influence of plaque-associated periodontal inflammation are necessary to clarify the direct effect of CsA. We have recently found CsA upregulates the expression of proliferating cell nuclear antigen (PCNA) and inducible nitric oxide synthase (iNOS) in gingival tissue of rats (11,12). Proliferating cell nuclear antigen is an antigen which has been widely used as a marker of cell proliferation, while iNOS is known to be associated with tissue inflammation (13–17). However, whether ET-1 plays a particular role in these associated activities during CsA therapy is still unknown. The aims of this study were: (1) to examine *in vivo* the effect of CsA on the expression of mRNA for ET-1, ET_A, ET_B, PCNA and iNOS in the edentulous gingiva of rats, where there is no gingival sulcus to facilitate accumulation of dental bacterial plaque; and (2) to evaluate *in vitro* the role of ET-1 by blockade of ET_A or ET_B in the CsA-associated induction of PCNA and iNOS expression.

Material and methods

Animal study

Maxillary edentulous ridges were established in 32 5-week-old male Sprague–Dawley rats weighing 120–150 g, as previously described (18,19). In brief, all right maxillary molars were extracted under general anesthesia with ketamine. After a 3 week healing period, the rats were randomly assigned to CsA and control groups. Animals received 30 mg/kg/day CsA (Sandimmun, Sandoz, Basel, Switzerland) via gastric feeding for 4 weeks, and rats in the control group received mineral oil via gastric feeding. At the end of study, all animals were killed by carbon dioxide inhalation. All the procedures were carried out with the approval of the Institutional Animal Care and Use Committee of the National Defense Medical Center. The gingival and surrounding mucosa of the edentulous ridges were immediately frozen in liquid nitrogen and stored at −80°C. Half the specimens were prepared for reverse transcription-polymerase chain reaction (RT-PCR) and the remaining specimens were transferred to prechilled 2-methylbutane for cryostat sectioning. The 5 μm-thick serial sections were sliced buccopalatally, and stored at −80°C for immunohistochemical (IHC) analysis.

Culture of human gingival fibroblasts and the *in vitro* study

Human gingival fibroblasts were prepared as described elsewhere (20). In brief, gingival specimens were obtained from the distal wedges during a flap operation on maxillary retromolar regions of patients undergoing periodontal surgeries at the Dental Clinic of Tri-Service General Hospital, Taipei, Taiwan. The gingival specimens were immersed for 2 days at 4°C in Leibovitz L-15 medium (Sigma-Aldrich Inc., St Louis, MO, USA) with 2 mg/mL dispase II (Roche Diagnostics, Indianapolis, IN, USA) and 10% fetal bovine serum (FBS). After separation from the outer epithelial layer, the connective tissue was minced and digested for 24 h in medium containing 10% FBS and 2 mg/mL collagenase (Sigma-Aldrich). The fragments were then placed in flasks with 10% FBS in DMEM/F-12 media to allow the cells to migrate from the explants. In all of the *in vitro* experiments, fibroblasts from the third passage that had reached 70% confluence in serum-free medium were used. The expression of mRNA for ET-1, ET_A and ET_B in harvested gingival fibroblasts stimulated for 24 h with CsA (Sigma-Aldrich) at final concentrations from 10 to 10^4 ng/mL in dimethyl sulphoxide (DMSO; Sigma-Aldrich) was determined by RT-PCR.

In this study, the expression of PCNA was selected as the indicator for cell proliferation (11). After being stimulated with CsA (0, 100, 500 and 1000 ng/mL in DMSO) for 24 h, fibroblasts were fixed with 4% paraformadehyde and stained with anti-PCNA antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) by immunocytochemistry (ICC). When applicable, cells were pretreated for 30 min before CsA treatment with 100 nM BQ-123 or BQ-788 (Sigma-Aldrich), selective antagonists for ET_A and ET_B, respectively (21,22). The percentages of cells positively stained for PCNA were counted in six selected areas per culture well. In addition, the expression of iNOS mRNA in gingival fibroblast cultures during CsA treatment following receptor antagonist pretreatment was evaluated by RT-PCR.

Extraction of RNA and reverse transcription–polymerase chain reaction

Total RNA was extracted from homogenized gingival tissue of rats or homogenized human fibroblast cultures. The RNA was reverse transcribed, and the PCR reactions included an initial denaturation at 94°C for 2 min 30 s, followed by the denaturation cycles between 30 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 40 cycles for others at 94°C for 30 s, annealing at

58–60°C for 30 s, and elongation at 72°C for 55 s. The PCR primers used in the present study were as follows: for rat samples, ET-1 (23), 5′-CGTCCCGTATGGACTAGGAA-3′ and 5′-TGCAACTCGAAAGGAGGTCT-3′; ET_A (23), 5′-ATCGGGATCCCCTTGATTAC-3′ and 5′-TGACAACCAAGCAGAAGACG-3′; ET_B (23), 5′-GGACTACAAAGGAAAGCCCC-3′ and 5′-TGCTCACCAAATACAGAGCG-3′; iNOS (24), 5′-CATTGCATTCAGATCCCGA-3′ and 5′-TGGGAGGGGTAGTGATGT-3′; PCNA (25), 5′-GCCCTCAAAGACCTCATCAA-3′ and 5′-GCTCCCCACTCGCAGAAAAC-3′; GAPDH (26), 5′-TGCTGGTGCTGAGTATGTCG-3′ and 5′-ATTGAGAGCAATGCCAGCC-3′; and for human gingival fibroblast samples, ET-1 (27), 5′-AGAGTGTGTCTACTTCTGCC-3′ and 5′-GCGTTATGTGACCCACAAC-3′; ET_A (27), 5′-CGCTGATAGCCAGTCTTG-3′ and 5′-CGGTTCTTGTCCATTTCG-3′; ET_B (27), 5′-GGACCCATCGAGATCAAGG-3′ and 5′-AGAATCCTGCTGAGGTGAAGG-3′; iNOS (28), 5′-GGCCTCGCTCTGGAAAGA-3′ and 5′-TCCATGCAGACAACCTT-3′; GAPDH (29), 5′-AGCCGCATCTTCTTTTGCGTC-3′ and 5′-TCATATTTGGCAGGTTTTTCT-3′. Amplified RT-PCR products were then analysed on a 1% agarose gel and visualized with ethidium bromide staining using a camera system (Transilluminator/SPOT; Diagnostic Instruments, Sterling Heights, MI, USA). The gel images were directly scanned (ONE-Dscan 1-D Gel Analysis Software; Scanalytic Inc., Fairfax, VA, USA), and the relative intensities were obtained by determining the ratio of the signal intensity to that of the GAPDH band.

Enzyme-linked immunosorbent assay (ELISA)

For quantification of ET-1 release, serum-free media from the human gingival fibroblast cultures were collected after 24 h of CsA treatment (0, 100, 500 and 1000 ng/mL in DMSO), centrifuged at 10,000*g* for 5 min to remove cellular debris, and ET-1 concentrations determined using an endo-

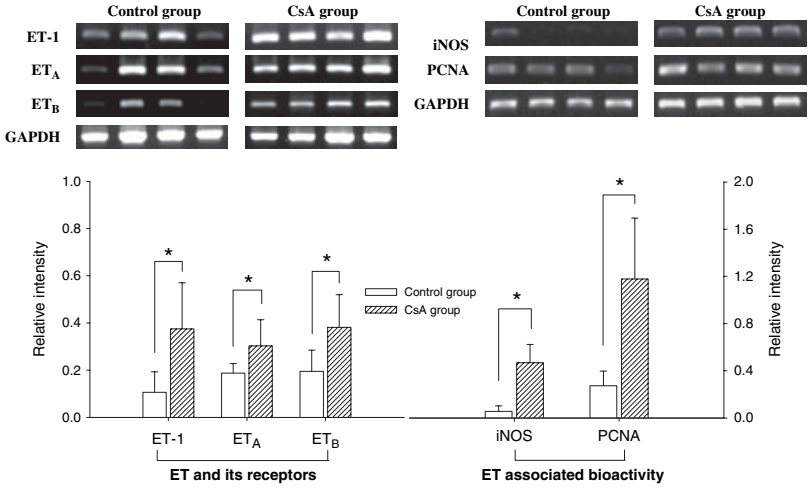

Fig. 1. Expression of mRNAs for ET-1, ET_A and ET_B, as well as PCNA and iNOS, in the gingival tissue of edentulous ridges from control and CsA-treated rats. The top part of the figure shows expression of mRNA in gingival tissue selected randomly from four rats in each animal group. The lower part of the figure shows a comparison of the relative intensities of mRNA for ET-1, ET_A and ET_B, as well as PCNA and iNOS, relative to GAPDH between the gingival tissues from the control and CsA-treated groups. (Means and standard deviations; *significant difference at $p < 0.05$).

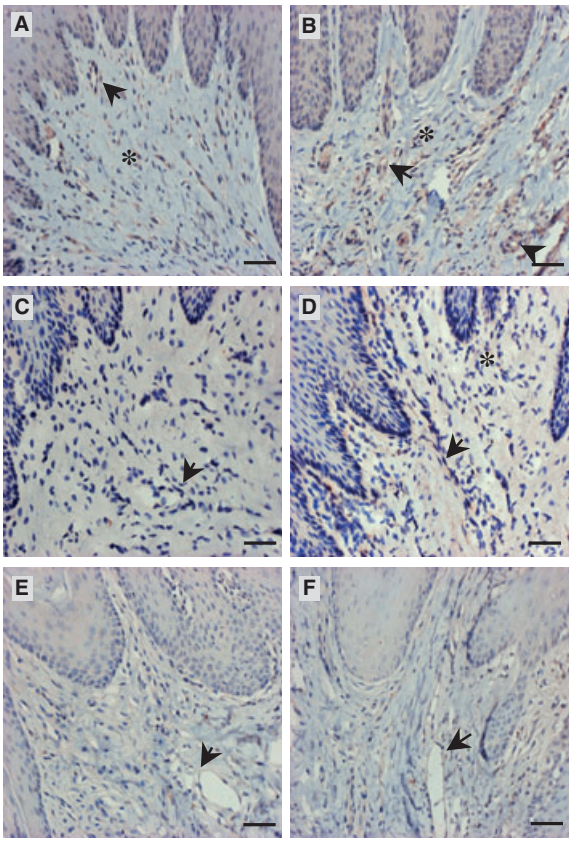

Fig. 2. Immunohistochemistry for ET-1 (A and B), ET_A (C and D) and ET_B (E and F) in the edentulous gingival tissue sections from the control (A, C and E) and CsA-treated rats (B, D and F). (*, fibroblasts; arrow, endothelial cells; arrowhead, smooth muscle cells; scale bar represents 50 μm).

thelin-1 chemiluminescent ELISA system (R&D Systems, Minneapolis, MN, USA). Cell counts were obtained by Coulter counter, and ET-1 concentrations were normalized to cell number.

Immunohistochemistry (IHC) and immunocytochemistry (ICC)

In this study, IHC was used to evaluate the expression of ET-1, ET_A and ET_B proteins in gingival tissue obtained from the animal study, whereas ICC was selected to examine PCNA expression in cultured gingival fibroblasts. After acetone fixation, the sections/fibroblasts were incubated with 0.1% hydrogen peroxide in distilled water to quench their endogenous peroxidase activity. The sections/fibroblasts were incubated for 2 h with unconjugated primary polyclonal antibodies against ET-1 (goat IgG; Calbiochem, San Diego, CA, USA), ET_A (rabbit IgG), ET_B (rabbit IgG; Abcam, Cambridge, MA, USA), or PCNA (Santa Cruz Biotechnology, Inc.), followed by further incubation with biotinylated secondary antibody for 30 min, streptavidin-conjugated horseradish peroxidase for 30 min, and 3-amino-9-ethyl carbazole solution for 10 min. The specimens were then counterstained with haematoxylin and mounted. Cells positively stained for ET-1, ET_A, ET_B and PCNA were examined under the microscope.

Statistical analysis

Student's *t*-test was used to evaluate the differences in the expression (the relative intensities) of mRNA for ET-1, ET_A, ET_B, PCNA and iNOS between animals from the control and CsA groups. One-way ANOVA was selected to evaluate the effect of CsA dose on the *in vitro* expression of mRNAs for ET-1, ET_A and ET_B, as well as the release of ET-1 protein, whereas two-way ANOVA was selected to evaluate the expression of iNOS mRNA after blockade of endothelin receptors followed by treatment with various concentrations of CsA. To examine the effect of different doses of CsA on ET-1 protein and iNOS mRNA expression, regression analysis was used. Repeated-measures ANOVA was used to examine the effects on the expression of PCNA in fibroblasts of CsA concentration and of ET-1 blockade (between-subject factors), as well as the variation in the six areas examined from each culture (within-subject factor). Duncan's test was used for *post hoc* analysis, and $p < 0.05$ was deemed to be significant.

Results

The expression of mRNA for ET-1, ET_A, ET_B, PCNA and iNOS appeared significantly greater in the edentulous gingiva of CsA-treated rats than in

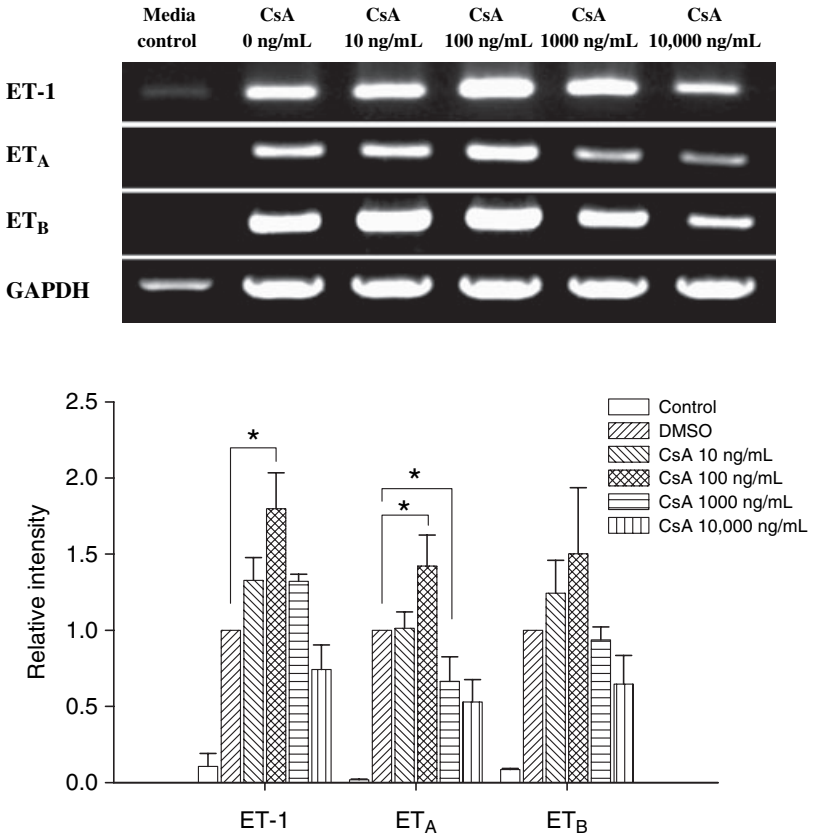

Fig. 3. The top part of the figure shows the expression level of mRNA for ET-1, ET_A and ET_B in human gingival fibroblast cultures after CsA treatments for 24 h. The lower part of the figure shows a comparison of the relative densities of mRNA for ET-1, ET_A and ET_B relative to GAPDH mRNA among the fibroblasts treated with different concentrations of CsA. (Means and standard deviations; *significant difference at $p < 0.05$).

those of the control animals (Fig. 1). Immunohistochemistry revealed that more cells, including endothelial cells, smooth muscle cells and fibroblasts, were positively stained for ET-1, ET_A and ET_B in the edentulous gingiva of CsA-treated rats than in those of control rats (Fig. 2).

In gingival fibroblast cultures, bimodal mRNA expression of ET-1, ET_A and ET_B was observed after CsA treatment when compared with control (DMSO-treated) cells. The mean level of mRNA expression of ET-1, ET_A and ET_B increased at CsA concentrations of 10 and 100 ng/mL (with statistical significance at 100 ng/mL of CsA for ET-1 and ET_A), whereas the mean expression reduced at high CsA concentration (with statistical significance at 1000 ng/mL of CsA for ET_A; Fig. 3). Dimethyl sulphoxide solvent appeared to enhance the mRNA expression of ET-1, ET_A and ET_B in fibroblast cultures when compared with the media control. A dose-dependent increase of ET-1 protein release ($p = 0.009$ and $R = 0.427$) was found in fibroblasts after CsA treatments (Fig. 4). Significantly increased ET-1 protein expression was observed with a CsA concentration of 1000 ng/mL than with any other concentration of CsA by Duncan's *post hoc* analysis after one-way ANOVA.

The percentages of PCNA-positive cells increased significantly in CsA-treated fibroblasts compared with those without CsA, regardless of the CsA concentration (100, 500 and 1000 ng/mL; Fig. 5A and left panel of Fig. 5C). In contrast, the percentage increase in stained cells was reduced at all CsA concentrations examined when cells were pretreated with ET_A antagonist (BQ-123), but not ET_B antagonist (BQ-788; Fig. 5B and right panel of Fig. 5C).

A dose-dependent increase of iNOS mRNA expression was found in fibroblasts after CsA treatments ($p < 0.001$ and $R = 0.879$), and a significant increase at the 1000 ng/mL CsA was observed when compared with that at 0 ng/mL by Duncan's *post hoc* analysis after one-way ANOVA (Fig. 6, left). After pretreating with BQ-788, a significant reduction in the induced iNOS expression was observed at the concentration of 1000 ng/mL, but the reduction was not noted after pretreating with BQ-123 (Fig. 6, right).

Discussion

In this study, an *in vivo* model, the edentulous gingivae of rat, and an *in vitro* model, primary human gingival fibroblast cultures, were selected and used to probe the role of ET-1 and its receptors in the development of CsA-induced gingival overgrowth. There are three known isoforms of endothelin (endothelin-1, endothelin-2 and endothelin-3), which are produced by endothelin-converting enzymes from biologically inactive intermediates via proteolytic processing at Trp-21-Val/Ile-22 (30,31). In mammals, there are

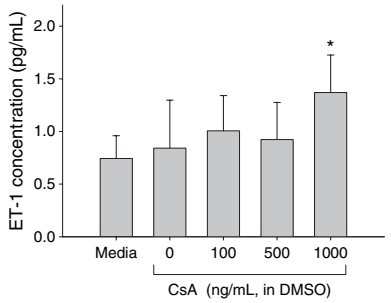

Fig. 4. Endothelin-1 release from the fibroblast cultures after CsA treatments for 24 h, measured by ELISA. (Means and standard deviations; * significant difference from any concentration of CsA by Duncan's *post hoc* analysis after one-way ANOVA; $R = 0.427$ and $p = 0.009$ by regression).

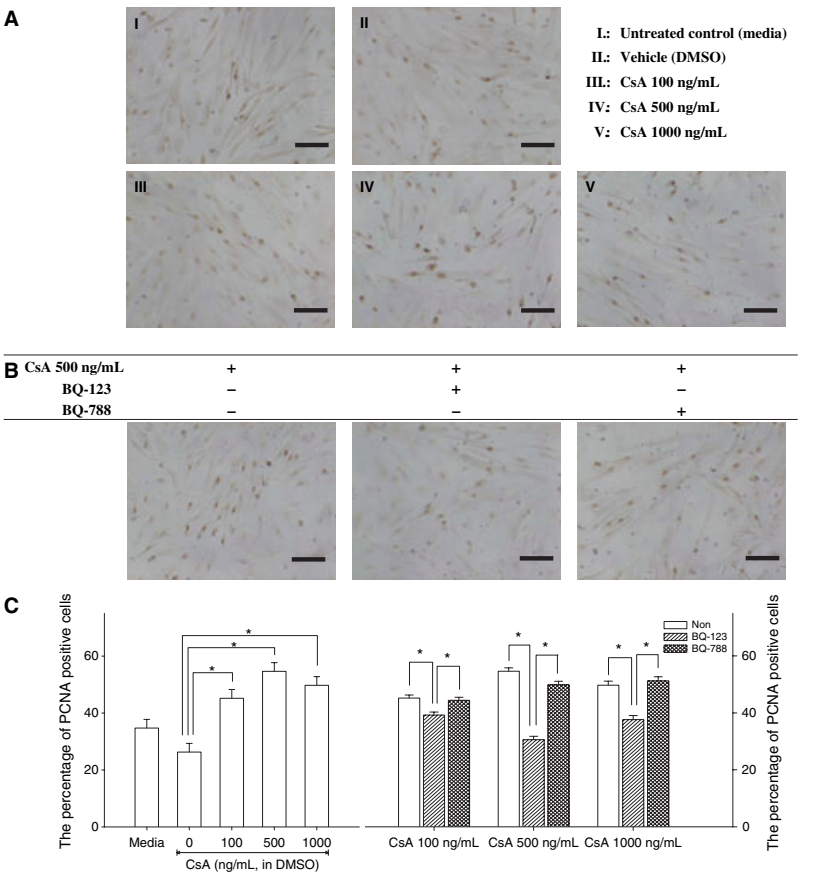

Fig. 5. The effect of ET-1 in the expression of PCNA in gingival fibroblast cultures after CsA treatment, assessed by immunocytochemistry. The photomicrographs in the A and B show the expression of PCNA-positive stained fibroblasts in the cultures after the treatment with CsA (A), as well as after blockade with ET-1 antagonists (B: +, with CsA, BQ-123 or BQ-788; –, without the treatment; scale bars represent 50 μm). C summarizes the effects of CsA treatment (left panel) and ET-1 antagonists (right panel) on the expression (percentages) of the positively stained cells in the cultures.

two G-protein-coupled endothelin receptors, ET_A and ET_B, each of which contains seven transmembrane domains of 22–26 hydrophobic amino acids within their approximately 400 amino acid sequences and are expressed in a wide variety of tissues (32–34). The ET_A receptor is selective for ET-1, but the ET_B receptor does not distinguish among the three isoforms of ET. Endothelin-1 demonstrates a wide range of biological properties, including significant mitogenic activity (4). In human lung fibroblasts, the ability of ET-1 to induce the expression of matrix metalloprotease-1 (MMP-1) mRNA and connective tissue growth factor protein was abrogated after blockade of the MAPK/ERK kinase pathway (35). In cultured rabbit gingival fibroblasts, ET-1 stimulated ERK1/2 activation via the ET_A receptor and led to cell proliferation (36). In the present study, the CsA-mediated enhancement of PCNA expression in gingival fibroblasts was partly abrogated by blockade of ET_A, suggesting that ET_A may contribute to cell proliferation and the development of gingival overgrowth.

Endothelin-1 has a dual effect on vascular tissue: it mediates vasoconstriction via ET_A activation of vascular smooth muscle cells and vasorelaxation via ET_B activation of endothelial cells through nitric oxide (NO) synthesis (8). In our recent studies, an increased expression of iNOS in gingiva of rats treated with CsA was observed, and the administration of both NO substrate (L-arginine) and NO blocking agent (*N*-nitro-L-arginine methylester-hydrochloride, L-NAME) could reduce the degree of gingival overgrowth (12,37). Although the exact mechanisms of this pathological gingival overgrowth are still uncertain, the present study showed that pretreatment with an ET_B antagonist, BQ-788, reduced the enhanced iNOS expression seen at a CsA concentration of 1000 ng/mL. We consequently suggest that ET_B may be partly involved in the expression of iNOS in gingiva during CsA treatment.

In the present study, the dose effect of CsA on the expression of ET-1, ET_A and ET_B in gingival fibroblasts was tested. A biphasic mRNA expression was observed in cells after treatment with varied concentrations of CsA when compared with those treated with DMSO (Fig. 3). The exact reason is unknown, but a similar pattern of a dual effect has also been observed on an induced cell apoptosis in endothelial cells after CsA treatment (38). In that study, low-dose CsA significantly reduced the induced apoptosis, whereas used at a high dose CsA did not reduce the induced apoptosis and even had deleterious effects. In our study, the mean level of mRNA expression of ET-1, ET_A and ET_B increased at CsA concentrations of 100 ng/mL (with statistical significance for ET-1 and ET_A) but declined at CsA concentrations of 10,000 ng/mL. Moreover, a study showed that the oral therapeutic dose of 10–20 mg/kg of body weight per day resulted in a serum concentration of 100–400 ng/mL, while the clinical gingival overgrowth could be easily observed in patients taking that therapeutic dose (39). One hundred and 500 ng/mL of CsA were, therefore, chosen to be the main concentrations used in the experiments on PCNA and iNOS expression.

Recently, we and other laboratories have reported an overexpression of vascular endothelial growth factor (VEGF) in gingivae of rats that had received CsA (40,41). Vascular endothelial growth factor is involved not only in angiogenesis, but also in wound healing and interstitial matrix remodelling (42). A potential interaction

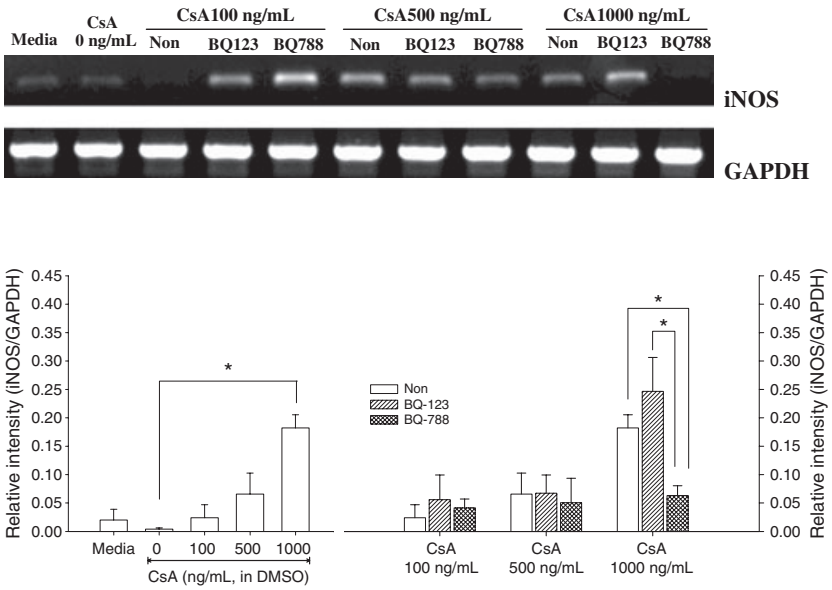

Fig. 6. The effect of ET-1 on mRNA expression for iNOS in gingival fibroblast cultures after CsA therapy. The top part of the figure shows the mRNA expression of iNOS in fibroblasts after CsA therapy, with/without ET-1 antagonists, while the lower part of the figure summarizes the effects of CsA treatment (left panel) and ET-1 antagonists (right panel) on iNOS mRNA expression. (Means and standard deviations; *significant difference at $p < 0.05$).

between VEGF and ET-1 has also been reported, in that VEGF enhanced both the expression of ET-1 mRNA and the secretion of ET-1 in endothelial cells (43). Similarly, in vascular smooth muscle cells, acting predominantly through the ET_A receptor, ET-1 enhanced both the expression of VEGF mRNA and the secretion of VEGF, and stimulated VEGF-induced endothelial cell proliferation and invasion (44,45). These observations indicate that VEGF and ET-1 have reciprocal stimulatory interactions that result in concomitant proliferation of endothelial cells and vascular smooth muscle cells. Studies have also shown that ET-1 promotes the contractile ability of normal fibroblasts (46) and modifies extracellular matrix metabolism, such as enhancing collagen types I and III and decreasing mRNA and protein expression of MMP-1 in dermal fibroblasts (6,7). Nevertheless, the exact mechanism leading to the overgrowth is still uncertain and requires further exploration.

In conclusion, the present results show that: (1) the expression of ET-1 and its receptors in gingiva are upregulated during CsA treatment in both *in vivo* and *in vitro* models; and (2) ET_A and ET_B have different bioactivities, in that ET_A appears to be principally involved in cell proliferation and ET_B seems to be associated with iNOS expression in the *in vitro* model. There is a high chance that ET-1 plays an important role in the pathogenesis of CsA-induced gingival overgrowth.

Acknowledgements

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References

1. Schincaglia GP, Forniti F, Cavallini R, Piva R, Calura G, del Senno L. Cyclosporin-A increases type I procollagen production and mRNA level in human gingival fibroblasts *in vitro*. *J Oral Pathol Med* 1992;**21:**181–185.
2. Zebrowski EJ, Pylypas SP, Odlum O, Johnson RB. Comparative metabolism of ^{3}H-glucosamine by fibroblast populations exposed to cyclosporine. *J Periodontol* 1994;**65:**565–567.
3. Yanagisawa M, Kurihara H, Kimura S *et al*. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;**332:**411–415.
4. Levin ER. Endothelins. *N Engl J Med* 1995;**333:**356–363.
5. Ito H, Hirata Y, Adachi S *et al*. Endothelin-1 is an autocrine/paracrine factor in the mechanism of angiotensin II-induced hypertrophy in cultured rat cardiomyocytes. *J Clin Invest* 1993;**92:** 398–403.
6. Shi-Wen X, Denton CP, Dashwood MR *et al*. Fibroblast matrix gene expression and connective tissue remodeling: role of endothelin-1. *J Invest Dermatol* 2001;**116:** 417–425.
7. Xu S, Denton CP, Holmes A, Dashwood MR, Abraham DJ, Black CM. Endothelins: effect on matrix biosynthesis and proliferation in normal and scleroderma fibroblasts. *J Cardiovasc Pharmacol* 1998; **31**(suppl 1)**:**S360–S363.
8. Marasciulo FL, Montagnani M, Potenza MA. Endothelin-1: the yin and yang on vascular function. *Curr Med Chem* 2006; **13:**1655–1665.
9. Tamilselvan S, Raju SN, Loganathan D, Kamatchiammal S, Abraham G, Suresh R. Endothelin-1 and its receptors ET_A and ET_B in drug-induced gingival overgrowth. *J Periodontol* 2007;**78:**290–295.
10. Yamamoto E, Awano S, Koseki T, Ansai T, Takehara T. Expression of endothelin-1 in gingival epithelial cells. *J Periodontal Res* 2003;**38:**417–421.
11. Chin YT, Chen YT, Tu HP *et al*. Upregulation of the expression of epidermal growth factor and its receptor in gingiva upon cyclosporin A treatment. *J Periodontol* 2006;**77:**647–656.
12. Gau CH, Chou TC, Chiu HC *et al*. Effect of cyclosporin A on the expression of inducible nitric oxide synthase in the gingiva of rats. *J Periodontol* 2005;**76:**2260–2266.
13. Beresford MJ, Wilson GD, Makris A. Measuring proliferation in breast cancer: practicalities and applications. *Breast Cancer Res* 2006;**8:**216.
14. Tachibana KE, Gonzalez MA, Coleman N. Cell-cycle-dependent regulation of DNA replication and its relevance to cancer pathology. *J Pathol* 2005;**205:**123–129.
15. Chen HI, Chang HR, Wu CY *et al*. Nitric oxide in the cardiovascular and pulmonary circulation – a brief review of literatures and historical landmarks. *Chin J Physiol* 2007;**50:**43–50.
16. Sastre M, Klockgether T, Heneka MT. Contribution of inflammatory processes to Alzheimer's disease: molecular mechanisms. *Int J Dev Neurosci* 2006;**24:**167–176.
17. Tinker AC, Wallace AV. Selective inhibitors of inducible nitric oxide synthase: potential agents for the treatment of inflammatory diseases? *Curr Top Med Chem* 2006;**6:**77–92.
18. Fu E, Hsieh YD, Shen EC, Nieh S, Mao TK, Chiang CY. Cyclosporin-induced gingival overgrowth at the newly formed edentulous ridge in rats: a morphological and histometric evaluation. *J Periodontol* 2001;**72:**889–894.
19. Gau CH, Hsieh YD, Shen EC, Lee S, Chiang CY, Fu E. Healing following tooth extraction in cyclosporine-fed rats. *Int J Oral Maxillofac Surg* 2005;**34:**782–788.
20. Chiang CY, Chen YT, Hung FM, Tu HP, Fu MM, Fu E. Cyclosporin-A inhibits the expression of cyclooxygenase-2 in gingiva. *J Periodontal Res* 2007;**42:**443–449.
21. Ishikawa K, Ihara M, Noguchi K *et al*. Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. *Proc Natl Acad Sci USA* 1994;**91:**4892–4896.
22. Warner TD, Allcock GH, Vane JR. Reversal of established responses to endothelin-1 *in vivo* and *in vitro* by the endothelin receptor antagonists, BQ-123 and PD 145065. *Br J Pharmacol* 1994; **112:**207–213.
23. Hirata Y, Ishimaru S. Effects of endothelin receptor antagonists on endothelin-1 and inducible nitric oxide synthase genes in a rat endotoxic shock model. *Clin Sci (Lond)* 2002;**103**(suppl 48)**:**332S–335S.
24. Yang AL, Tsai SJ, Jiang MJ, Jen CJ, Chen HI. Chronic exercise increases both inducible and endothelial nitric oxide synthase gene expression in endothelial cells of rat aorta. *J Biomed Sci* 2002;**9:**149–155.
25. Hur K, Kim JR, Yoon BI *et al*. Overexpression of cyclin D1 and cyclin E in 1,2-dimethylhydrazine dihydrochloride-induced rat colon carcinogenesis. *J Vet Sci* 2000;**1:**121–126.
26. Drissi H, Lomri A, Lasmoles F, Holy X, Zerath E, Marie PJ. Skeletal unloading induces biphasic changes in insulin-like growth factor-I mRNA levels and osteoblast activity. *Exp Cell Res* 1999;**251:**275–284.
27. Fujioka D, Nakamura S, Yoshino H *et al*. Expression of endothelins and their receptors in cells from human periodontal tissues. *J Periodontal Res* 2003;**38:**269–275.
28. Smith RE, Robinson NM, McPeake JR *et al*. Induction and role of NO synthase in hypotensive hepatic failure. *Arterioscler Thromb Vasc Biol* 1997;**17:**3079–3082.
29. Miura M, Gronthos S, Zhao M *et al*. SHED: stem cells from human exfoliated

deciduous teeth. *Proc Natl Acad Sci USA* 2003;**100:**5807–5812.
30. Rubanyi GM, Botelho LH. Endothelins. *FASEB J* 1991;**5:**2713–2720.
31. Anggard EE, Botting RM, Vane JR. Endothelins. *Blood Vessels* 1990;**27:**269–281.
32. Lin HY, Kaji EH, Winkel GK, Ives HE, Lodish HF. Cloning and functional expression of a vascular smooth muscle endothelin 1 receptor. *Proc Natl Acad Sci USA* 1991;**88:**3185–3189.
33. Sakurai T, Yanagisawa M, Takuwa Y *et al*. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* 1990;**348:**732–735.
34. Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev* 1994;**46:**325–415.
35. Xu SW, Howat SL, Renzoni EA *et al*. Endothelin-1 induces expression of matrix-associated genes in lung fibroblasts through MEK/ERK. *J Biol Chem* 2004;**279:**23098–23103.
36. Ohsawa M, Ohuchi N, Taniguchi Y *et al*. Inhibition of angiotensin II- and endothelin-1-stimulated proliferation by selective MEK inhibitor in cultured rabbit gingival fibroblastsdagger. *Fundam Clin Pharmacol* 2005;**19:**677–685.
37. Fu E, Tz-Chong C, Liu D, Chiu SC. Ameliorated effect of L-arginine supplementation on gingival morphology in cyclosporin-treated rats. *J Periodontol* 2000;**71:**1737–1742.
38. Mailloux A, Bruneel A, Vaubourdolle M, Baudin B. Cyclosporin A but not estradiol can protect endothelial cells against etoposide-induced apoptosis. *Endothelium* 2004;**11:**141–149.
39. Boltchi FE, Rees TD, Iacopino AM. Cyclosporine A-induced gingival overgrowth: a comprehensive review. *Quintessence Int* 1999;**30:**775–783.
40. Cetinkaya BO, Acikgoz G, Ayas B, Aliyev E, Sakallioglu EE. Increased expression of vascular endothelial growth factor in cyclosporin A-induced gingival overgrowth in rats. *J Periodontol* 2006;**77:**54–60.
41. Chen YT, Tu HP, Chin YT *et al*. Upregulation of transforming growth factor-β1 and vascular endothelial growth factor gene and protein expression in cyclosporin-induced overgrown edentulous gingiva in rats. *J Periodontol* 2005;**76:**2267–2275.
42. Schrijvers BF, Flyvbjerg A, De Vriese AS. The role of vascular endothelial growth factor (VEGF) in renal pathophysiology. *Kidney Int* 2004;**65:**2003–2017.
43. Matsuura A, Yamochi W, Hirata K, Kawashima S, Yokoyama M. Stimulatory interaction between vascular endothelial growth factor and endothelin-1 on each gene expression. *Hypertension* 1998;**32:**89–95.
44. Okuda Y, Tsurumaru K, Suzuki S *et al*. Hypoxia and endothelin-1 induce VEGF production in human vascular smooth muscle cells. *Life Sci* 1998;**63:**477–484.
45. Pedram A, Razandi M, Hu RM, Levin ER. Vasoactive peptides modulate vascular endothelial cell growth factor production and endothelial cell proliferation and invasion. *J Biol Chem* 1997;**272:**17097–17103.
46. Grinnell F. Fibroblasts, myofibroblasts, and wound contraction. *J Cell Biol* 1994;**124:**401–404.