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Decreased expressions of thrombospondin 2 in cyclosporin A-induced gingival overgrowth

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Objectives: Cyclosporin A (CsA) is known to elicit fibrous gingival overgrowth with changes of blood vessel profiles. In this study, we examined the expression of several angiogenic and angiostatic genes during the development of CsA-induced gingival overgrowth.

Methods: For the development of gingival overgrowth, Sprague-Dawley rats received subcutaneous injections of CsA in daily doses of 5, 10, 15 mg/kg body weight for 6 weeks, and another group received 10 mg/kg of CsA for 3, 6, and 12 weeks. Human gingival tissues were obtained from three CsA-treated patients following the gingivectomy procedure and from three healthy patients following the crown-lengthening procedure as a control. Gingival fibroblasts were isolated from the healthy gingival tissues of the rat or the human, and cultured with 250–1000 ng/ml of CsA.

Results: Reverse transcription–polymerase chain reaction (RT–PCR) analyses showed that expressions of some angiogenic genes such as angiopoietin 1, basic fibroblast growth factor, and vascular endothelial growth factor, and angiostatic genes such as angiopoietin 2, brain-specific angiogenesis inhibitor 1 and 2, and thrombospondin 1 were not changed significantly in both gingival tissues and cultured fibroblast cells under the CsA treatments. However, expression of thrombospondin 2 (TSP2) decreased dose- and time-dependently in rat and human gingival tissues. Western blot analyses showed that the expression of TSP2 protein was dose-dependently reduced by the CsA treatments in human cultured gingival fibroblasts.

Conclusions: These results indicate that the decrease in angiostatic TSP2 expression may be attributed to the CsA-induced gingival vascularization rather than to the increased expression of angiogenic genes. It suggests that TSP2 is involved in the development of CsA-induced gingival overgrowth with the gingival vascularization.

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Cyclosporin A (CsA) is an immunosuppressive drug to prevent rejection of transplanted organs and also to treat various autoimmune diseases such as Behçet disease, pemphigus, rheumatoid arthritis, psoriasis and other dermatological diseases (1). CsA binds to cytoplasmic receptors called cyclophillin (CyP), and the CsA–CyP complexes inhibit Ca^{2+}- and calmodulin-dependent protein phosphatase calcineurin (2). The action of CsA blocks interleukin-2 synthesis by CD4 lymphocytes and promotes suppression of the immune response (3). CsA treatment may cause various side-effects including nephropathy, hepatotoxicity, neurotoxicity, hypertension, and gingival overgrowth (4, 5).

CsA-induced gingival overgrowth mostly affects the attached gingiva and is initially observed as a papillary gingival enlargement, which is prominent on the labial aspects with a lobulated appearance (6). One of the prominent histological findings in the gingival overgrowth is enlargement of connective tissue and vascularization. According to previous histomorphometric studies, CsA-induced gingival overgrowth showed increases in the mean cross-sectional area of the free gingival tissue, the connective tissue, and the blood vessel profile (5, 7–10).

For the above histopathological changes in CsA-induced gingival overgrowth, biochemical events such as accumulation of collagen fibers, inhibition of cellular phagocytosis, and the inhibition of secretion and activation of matrix metalloproteinases have been reported (11, 12). However, to date there is no report about the biochemical findings underlying the change of blood vessel profile in CsA-induced gingival overgrowth.

Formation of new blood vessels is affected by a balance between angiogenic and angiostatic factors. Vascular endothelial growth factor (VEGF), angiopoietin 1 (AGP1), basic fibroblast growth factor (bFGF), and transforming growth factor-beta1, called angiogenic factors, have been reported to elicit the formation of blood vessels and influence the migration of endothelial cells in some cancer tissues and hypoxic conditions. On the contrary, angiopoietin 2 (AGP2), angiostatin, endostatin, brain-specific angiogenesis inhibitors (BAIs), and thrombospondins (TSPs), called angiostatic factors, have been described to inhibit the angiogenesis in *in vivo* and *in vitro* experiments (13). CsA has been reported to exert an inhibitory activity on the new blood vessel formation of a chicken embryo chollioallantoic membrane, which is induced by an implantation of rheumatic arthritis and osteoarthritis tissue (14). CsA also produces anti-proliferative and proapoptotic effects mediated by the caspase pathway in the microvascular endothelial cells derived from human skin (15). However, in gingival tissues, CsA brought about increases in histomorphometrical profiles of blood vessel with enlargements of epithelium and connective tissue (9, 10), but its biochemical findings are not fully understood. This present study was undertaken to examine what kinds of angiogenic and angiostatic genes are involved in the pathogenesis of CsA-induced gingival overgrowth.

Materials and methods

Rat and human gingival tissue preparation

For the development of gingival overgrowth, 12 Sprague-Dawley rats weighing approximately 50 g received subcutaneous injections of CsA (Sandimmun-Sandoz, Santo Amaro, SP, Brazil) in daily doses of 5, 10, 15 mg/kg body weight for 6 weeks with a control group of four rats, and another 12 rats received 10 mg/kg of CsA for 3, 6, and 12 weeks. The administered dosage of 10 mg/kg provides reproducible and steady CsA serum levels with peaks and trough levels of 750 and 1000 ng/ml, respectively (16). The rats were weighed every 3 days to adjust the CsA dosage, and the control animals received only sterile caster oil as a vehicle. At the end of the experimental periods, CsA-treated and control rats were killed by anesthetic overdose of pentobarbital sodium (50 mg/kg) and then gingival tissues were removed from the mandibular anterior teeth carefully. For histological confirmation of the CsA-induced gingival overgrowth, gingival tissues of CsA (10 mg/kg)-treated rats were fixed in 10% neutral buffered formalin, dehydrated in a graded ethanol series, and embedded in paraffin with control. Sections were cut to approximately 5 μm thick, and stained with hematoxylin and eosin.

Human gingival tissues were obtained from three healthy patients by the crown-lengthening procedure as control, and from three gingival overgrowth patients, who were taking CsA for 2 years following renal transplantation, by gingivectomy procedure. This protocol was approved from the ethnic committee of Chonnam University Hospital.

Cell isolation and culture

Rat gingival tissue was carefully isolated from the healthy attached gingiva of the lower anterior teeth. The isolated tissue was washed three times with phosphate-buffered saline, and then minced and incubated in 5 ml of alpha Minimal Essential Medium (α-MEM, Gibco-BRL, NY, USA) containing 50 μg/ml of gentamycin, 50 U/ml of penicillin G, and 50 μg/ml of streptomycin at 37°C for 3 days. Gingival cells, which were migrated from the minced tissue to its periphery, were treated with 0.05% trypsin-EDTA, and then harvested and passaged into new 25-cm^2 flasks (Corning, NY, USA). After seven or eight passages of the cells, only gingival fibroblasts remained. These isolated cells were seeded in new 25-cm^2 flasks at a density of 1×10^5 cells, and then treated with 250, 500, 750, or 1000 ng/ml of CsA for 4 days. For the time-dependent effects of CsA, gingival fibroblasts were cultured for 1, 2, 3, and 4 days under 1000 ng/ml of CsA treatment.

Human gingival fibroblasts were obtained from gingivectomized tissue of a healthy individual through the crown-lengthening procedure, who had no clinical signs of inflammation in the periodontal tissues (i.e. no visible plaque, periodontal probing depth < 3 mm, no bleeding on gentle probing, no signs of clinical loss of attachment). The human gingival cells were also isolated and cultured using the same method above.

RNA extraction and Reverse transcription–polymerase chain reaction (RT–PCR)

For extraction of total RNA, gingival tissues and cultured gingival fibroblasts obtained from control and CsA-treated groups of rat or human were homogenized with a polytron homogenizer in Trizol reagent (Gibco-BRL). RNA samples were quantified by spectrophotometry at 260 nm wavelength. Each cDNA was generated from 0.4 μg of total RNA with Superscript II (Gibco-BRL) at 42°C for 1 h and used as a template for PCR analyses. The RT–PCR exponential phase was

determined on 30–35 cycles to allow quantitative comparisons among the cDNAs developed from identical reactions. All reactions involved an initial denaturation at 94°C for 5 min followed by 30 or 35 cycles at 94°C for 50 s, exposure to an appropriate annealing temperature (50–63°C) for 50 s, and 72°C for 1 min 40 s, on a Mastercycler Personal PCR system (Eppendorf, Hamburg, Germany). After making the first strand cDNA by reverse transcription, control PCR was performed on 25–27 cycles to allow quantitative comparisons among the cDNAs developed from identical reactions with primers for the housekeeping gene, β-actin. After confirming the same quantity of β-actin bands among them by analyzing the samples on agarose gels, main PCR reactions were processed using specific primers for desired genes. PCR primer sequences for angiogenic (AGP1, bFGF, VEGF) and angiostatic (AGP2, BAI1, BAI2, TSP1, TSP2) genes are shown in Table 1. The amplified products were analyzed on agarose gels and visualized by Transilluminator/Polaroid camera system (UVP Laboratories, Upland, CA, USA) with ethidium bromide staining.

To quantify the expressions, gel images from RT–PCR products were scanned, and relative densities were obtained by Scion Imaging analyses program (Scion Corporation, Frederick, MD, USA). Expressions of the genes between CsA-treated and control groups were compared as the ratio of signal intensity to β-actin in each group.

Western blot analyses

The cultured human gingival fibroblasts were reacted with a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 tablet/10 ml of complete protease inhibitor mixture (Boehringer Mannheim, Indianapolis, IN, USA), and 1% v/v NP-40. The resolved proteins (50 μg) were electrophoresed and transferred to a nitrocellulose membrane. The membrane was incubated with Tris-buffered saline containing 0.05% Tween 20 (TBST, Sigma, St. Louis, USA) for 2 h, rinsed, and then incubated with monoclonal antibody to thrombospondin 2 (1 : 400 in TBST, Santa-Cruz, CA, USA) for 2 h. After three washes for 30 min, the membrane was incubated for 1 h with anti-goat horseradish peroxidase-conjugated antibody (1 : 10,000 in TBST, Sigma, USA), and washed again as the above. Using the enhanced chemiluminescence + western blotting detection system (Amersham Pharmacia Biotech, Little Chalfont, UK), TSP2 protein was visualized in high performance chemiluminescence hyperfilm (Amersham Pharmacia Biotech, UK). The expressional changes following the CsA treatment were compared with the densities of scanned images by Scion Imaging analysis program (Scion Corporation).

Statistical evaluation

The results are expressed as means ± SD from three or four experiments. Statistical differences between groups were determined using unpaired Student's *t*-test. When p is less than 0.05, the difference was considered significantly.

Table 1. Primers for angiogenic and angiostatic genes used in reverse transcription-polymerase chain reaction (RT–PCR)

Primer		Oligonucleotide sequences	Product size (bp)	Annealing temperature (°C)
Angiogenic				
AGP1	S	5′-ACTTGAGAATTACATTGTGG-3′	257	50
	AS	5′-GAATTTCATTTGTCTGTTGG-3′		
bFGF	S	5′-ACCCGGCCACTTCAAGGATC-3′	496	54
	AS	5′-CTCAGCATGGAGGAACTCCCTG-3′		
VEGF	S	5′-GCTCTCTTGGGTGCACTGGA-3′	644	59
	AS	5′-CACCGCCTTGGCTTGTCACA-3′		
Angiostatic				
AGP2	S	5′-TATTGGCTGGGCAACGAGTTTG-3′	309	60
	AS	5′-CAAGTTGGAAGGACCACATGCG-3′		
BAI1	S	5′-ATGACCGACTTCGAGAAGGACG-3′	460	62
	AS	5′-TCTGCGGCATCTGGTCAATGTG-3′		
BAI2	S	5′-GTGTCCAGCCTTCCATGAGATG-3′	452	62
	AS	5′-TTTCCGCATCCACCATGAAGC-3′		
TSP1	S	5′-CGTCCTGTTCCTGATGCATG-3′	472	61
	AS	5′-GGCCCTGTCTTCCTGCACAA-3′		
TSP2	S	5′-CTGTGTCAACACTCAGCCTGGC-3′	434	61
	AS	5′-TCCTTCTCATCGGTCACACCG-3′		
β-ACTIN	S	5′-GATGATATCGCCGCGCTCGTC-3′	530	60
	AS	5′-AGCCAGGTCCAGACGCAGGAT-3′		

S, sense; AS, anti-sense.

Results

Expressions of angiogenic and angiostatic genes in the gingival tissues of CsA-treated rats

To develop the gingival overgrowth, CsA was injected into rats in daily doses of 5, 10, 15 mg/kg body weight for 6 weeks, and also 10 mg/kg of CsA was injected for 3, 6, and 12 weeks. CsA at a dose of 10 mg/kg for 6 weeks induced a clinically evident gingival overgrowth affecting incisor regions at the interdental papilla, and produced a gradual tooth separation. Microscopic examination of the region revealed typical findings of CsA-induced gingival overgrowth, such as the deposition of collagen fibrils, enlargement of connective tissue, epithelial thickening, and prominent rete pegs (data not shown).

To determine the type of angiogenesis-related genes involved in the CsA-induced gingival overgrowth, RT–PCR analyses were performed using specific primers for the angiogenic (AGP1, bFGF, VEGF) and angiostatic (AGP2, BAI1, TSP1, TSP2) genes. Expressional changes of the above genes except for TSP2 in the gingival tissue of CsA-treated rat were not significantly changed over the control, but TSP2 expression markedly decreased in the CsA-treated gingival tissue (Fig. 1). The decrease in TSP2 expression was

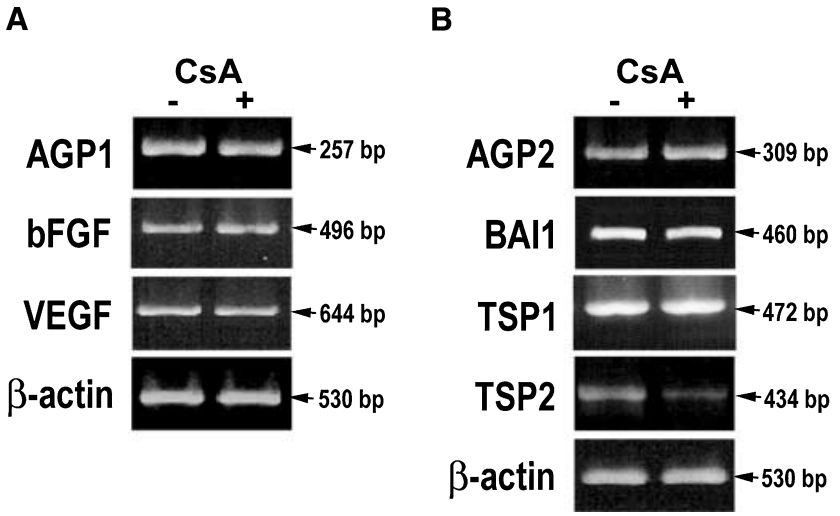

Fig. 1. Comparison of angiogenic (A) and angiostatic (B) gene expressions in cyclosporin A (CsA)-induced gingival overgrowth tissues of rats by reverse transcription-polymerase chain reaction analyses. CsA (10 mg/kg) was administered subcutaneously for 6 weeks. A representative gel image of four experiments is shown. Expressions of most angiogenesis-related genes are not changed, but expression of thrombospondin 2 mRNA is reduced. β-Actin is included as a control for confirming RNA fidelity. A thin arrow indicates each PCR amplification product. AGP, Angiopoietin; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; BAI, brain-specific angiogenesis inhibitor; TSP, thrombospondin.

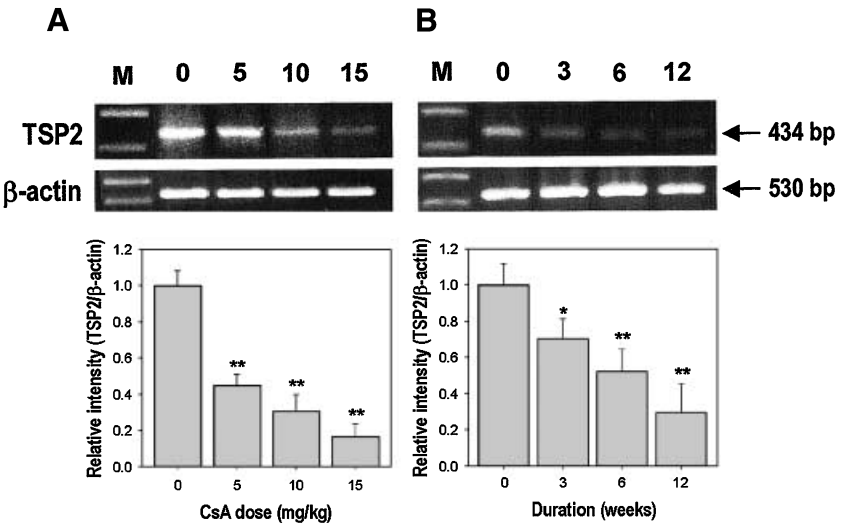

Fig. 2. Expressional changes of thrombospondin 2 (TSP2) mRNA by cyclosporin A (CsA) treatment in gingival tissues of rats. (A) CsA was injected subcutaneously with 5, 10, and 15 mg/kg for 6 weeks. Upper panel is a representative TSP2 expression of four experiments, and lower panel shows the normalized expressions of TSP2 to β-actin by densitometric analyses using the Scion Image analysis program. Expression of TSP2 mRNA by CsA decreased in a dose-dependent fashion. (B) Time-course effect of CsA on the expression of TSP2. CsA (10 mg/kg) was administered for 3, 6, and 12 weeks. TSP2 expression decreased time-dependently. Data are expressed as means ± SD from four experiments. Asterisks represent significant changes from the control value (*$p < 0.05$; **$p < 0.01$). M indicates a molecular marker.

dependent on the administered doses of CsA, ranging from 5 to 15 mg/kg intraperitoneally (Fig. 2A), and it also tended to decrease in a time-dependent manner over the 12-week period (Fig. 2B).

Changes of angiogenic and angiostatic genes in the rat cultured gingival fibroblasts

To determine whether the expressional changes of angiogenic and angiostatic genes noted in the gingival tissues also occurred in the gingival fibroblast cells, a cell potentially responsible for development of CsA-induced gingival overgrowth, RT–PCR analyses were also undertaken in primary cultured gingival fibroblasts from rats. Expression of angiogenic AGP1, bFGF, and VEGF was not changed by 1000 ng/ml of CsA, and none of the angiostatic genes such as AGP2, brain-specific angiogenesis inhibitors, and TSP1 were significantly changed. However, TSP2 expression prominently decreased compared to the control as noted in the *in vivo* gingival tissue. It is notable that TSP1 did not change, although its structure and actions are very similar to TSP2 (Fig. 3).

Changes of TSP2 expression by CsA in gingival tissues and gingival fibroblasts of human

To examine whether expressional changes of TSP2 induced by CsA are also produced in human gingival tissues and fibroblasts as in the rat, RT–PCR and western blot analyses were done on human samples. Human gingival tissues were obtained from three healthy patients by crown-lengthening procedure as controls, and from three CsA-treated renal-graft patients showing prominent gingival overgrowth by the gingivectomy procedure. The expressions of TSP2 mRNA in the CsA-treated gingival tissue decreased to 42 ± 7.2% of the control ($n = 3$, Fig. 4A). In the cultured gingival fibroblasts derived from the healthy gingival tissue, expression of TSP2 mRNA decreased dose-dependently, ranging from 250 to 750 ng/ml of CsA (Fig. 4B). And the expression also gradually decreased after one, two, three, and four days under the 1000 ng/ml of CsA treatments (Fig. 4C). Western blot analyses showed that expression of TSP2 protein was also reduced dose-dependently (Fig. 5).

Discussion

The relationship between CsA treatment and gingival overgrowth has been widely reported in human and some experimental models (1, 6, 9), although its underlying biochemical mechanisms are not completely understood yet. The pathophysiological mechanisms of CsA-induced gingival overgrowth have been suggested to include features such as an increase in the proliferation of fibroblasts and an abnormal accumulation of ECM components through the inhibition of degradation enzymes in the gingival tissue (10–12). Previous studies have shown that CsA increases in the cross-sectional area of blood vessel in gingival connective tissue (9, 10). The present study focused on the regulation of the expression of angiogenesis-related genes by CsA in gingival tissue and cultured gingival fibroblast cells. We found that the expression of TSP2 decreased in CsA-treated gingival tissues and fibroblasts, and that the decreased expression of angiostatic gene rather than an increased expression of angiogenic gene seemed to participate in increased vascularization during the development of CsA-induced gingival overgrowth.

VEGF and bFGF, mitogens for human dermal microvascular endothelial cells, are well known to induce the migration of endothelial cell *in vitro* (17, 18) and also to enhance microvascular permeability and angiogenesis

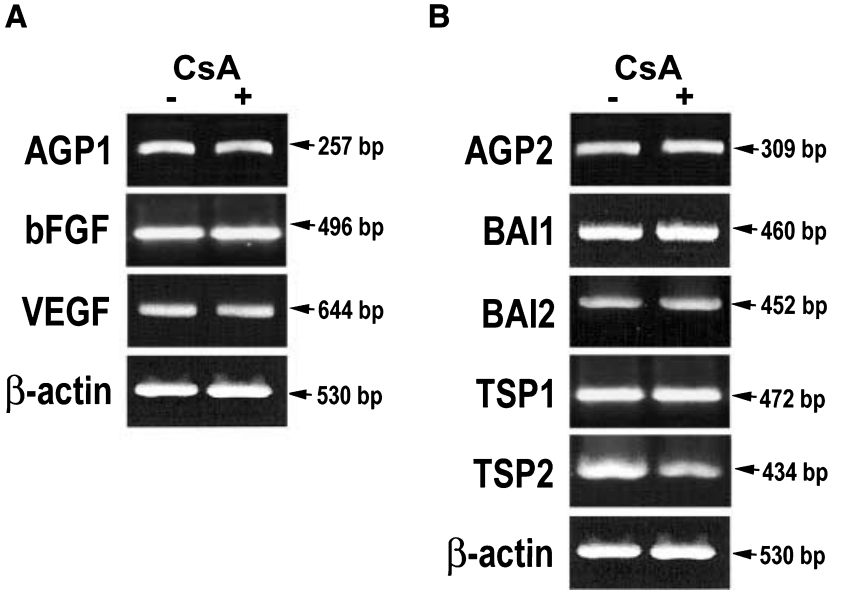

Fig. 3. Comparison of angiogenic (A) and angiostatic (B) gene expressions under 1000 ng/ml of cyclosporin A treatment for 4 days in the cultured gingival fibroblast derived from the rat. Expression of thrombospondin 2 mRNA is predominantly reduced; however, expressions of most of the genes, including thrombospondin 1, were not significantly changed. β-Actin is included as a control for confirming RNA fidelity. A thin arrow indicates each polymerase chain reaction amplification product. See Fig. 1 for details of abbreviations.

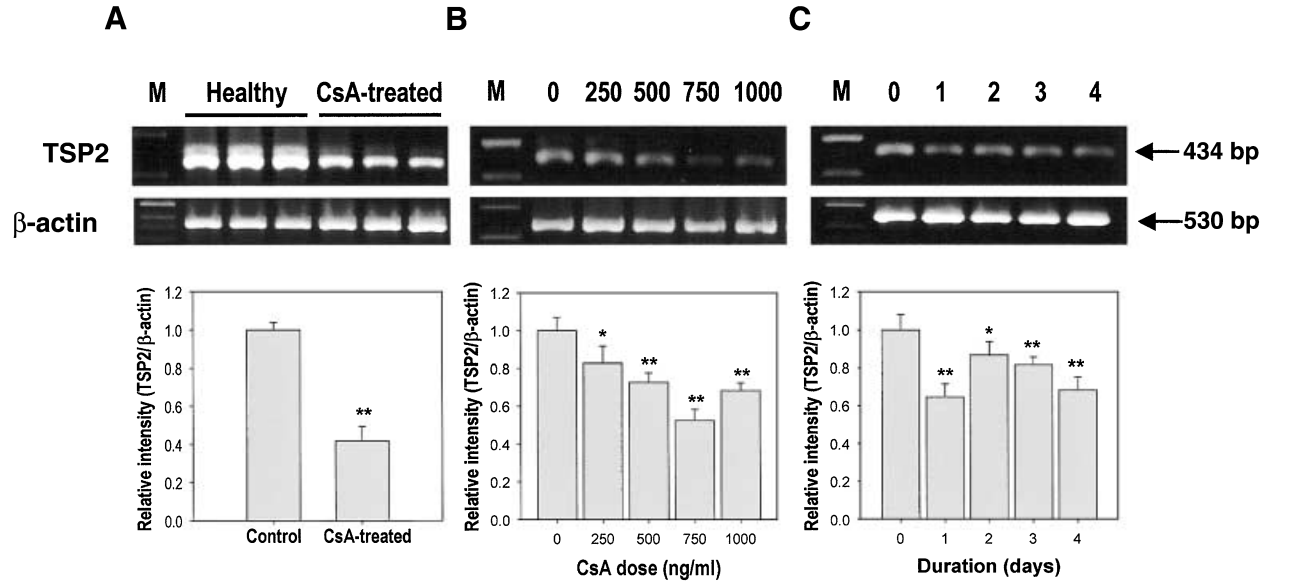

Fig. 4. Expressional changes of thrombospondin 2 (TSP2) mRNA in human gingival tissues (A) and cultured human gingival fibroblasts under the cyclosporin A (CsA) treatment. In three CsA-treated gingival overgrowth tissues, expressions of TSP2 decreased to 58% of three healthy gingival tissues, obtained by the crown-lengthening procedure. (B) Expressional changes of TSP2 mRNA under the treatment of CsA (250, 500, 750, and 1000 ng/ml) for 4 days in human cultured gingival fibroblasts. TSP2 expression decreased dose-dependently. (C) Expressional changes of TSP2 on 1, 2, 3, and 4 days after 1000 ng/ml of CsA treatment. TSP2 expression decreased time-dependently. Data are expressed as means ± SD from three experiments. Asterisks represent significant changes from the control value (*$p < 0.05$; **$p < 0.01$). M indicates a molecular marker.

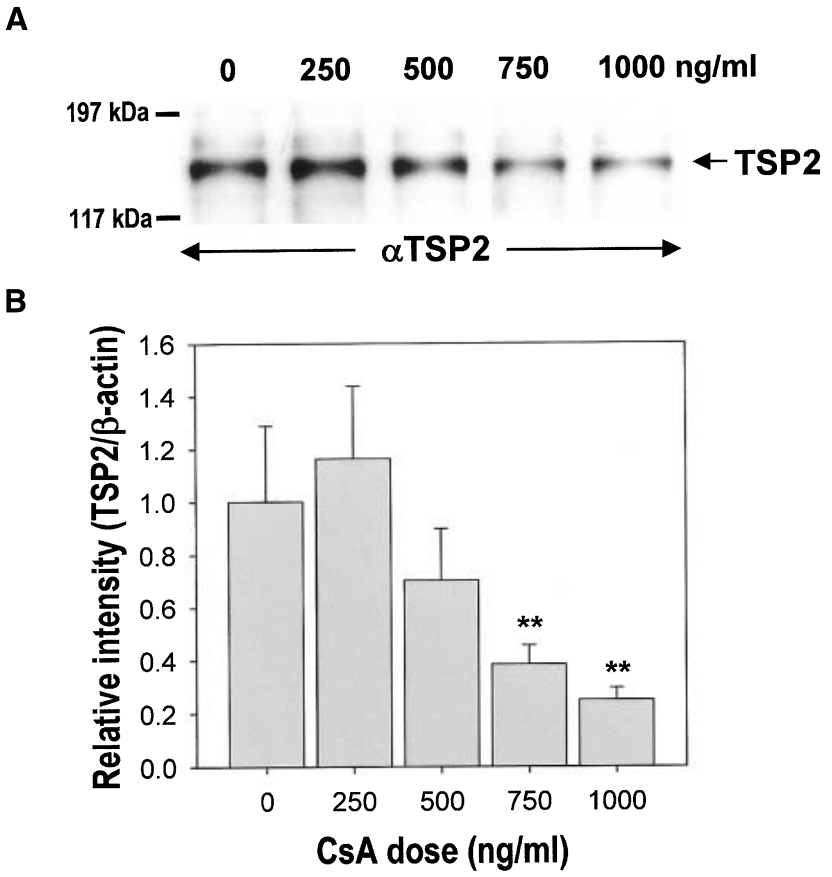

Fig. 5. Changes of thrombospondin 2 (TSP2) protein expression in cultured human gingival fibroblasts under the cyclosporin A (CsA) (1000 ng/ml) treatment. (A) A representative western blot analysis of three experiments. (B) Densitometric analyses of the TSP2 expression using the Scion Image analysis program. Note the reduced TSP2 expression along the increase in CsA concentration administered, ranging from 250 to 1000 ng/ml. Data are expressed as means ± SD from three experiments. Asterisks represent significant changes from the control value of TSP2 expression (**$p < 0.01$).

in vivo (19). These factors are involved in many diseases and drug-induced disorders which are accompanied with angiogenesis. For example, VEGF is expressed at low levels in normal skin, whereas skin diseases associated with the formation of blood vessel such as psoriasis, bullous disease, and squamous cell carcinoma show prominent up-regulations of VEGF expression (20). Pyogenic granuloma in oral gingival tissue also gives rise to up-regulation of VEGF and bFGF with increased blood vessel formation (21). Long-term administration of CsA elicited up-regulation of VEGF mRNA, protein, and VEGF receptors, Flt-1 and KDR/Flt-1, in nephrotoxic and fibrotic kidney (22). However, the drug also has anti-angiogenic effects to inhibit an *in vitro* migration of primary endothelial cells and an *in vivo* VEGF-induced corneal angiogenesis (23). In the present study, expressions of VEGF and bFGF mRNA were not affected in the CsA-induced gingival overgrowth tissue and CsA-treated fibroblasts. These results were different from the up-regulation of such factors observed in CsA-induced renal fibrosis and phenytoin-/nifedipine-induced gingival overgrowth accompanied with the increase of blood vessel formation (24, 25). However, there is one report that CsA did not affect the bFGF-induced angiogenesis in the cornea pocket of rabbit (23). These data indicate that VEGF and bFGF may not be a critical angiogenic regulator of CsA-induced gingival overgrowth, and that the pathogenesis of CsA-induced gingival overgrowth is not the same as gingival overgrowth induced by nifedipine and phenytoin.

Thrombospondins (TSP1 and TSP2) have an inhibitory activity against bFGF-induced experimental angiogenesis (26) and new blood vessel formation in cancer tissues such as bladder cancer, breast carcinoma, lung cancer, and colon cancer (20, 27–30). The angiostatic effects are closely related to the TSP-type 1 repeat domains of TSP1 and TSP2, and they elicit an inhibition of tumor formation (26). Although it differs from experimental systems, the angiostatic effect of TSP2 is considered to be more potent than that of TSP1 because TSP2-deficient mice show a more dramatic increase in formation of blood vessels than TSP-1 deficient mice (31). Meanwhile, the vasculature of normal adult skin remains quiescent, due to the dominant influence of endogenous angiogenesis inhibitors like TSPs over angiogenic stimuli, and down-regulation of such peptides produces a brisk angiogenesis during tissue repair and in numerous diseases such as psoriasis and squamous cell carcinomas (20). In our study, the expressions of angiostatic TSP2 prominently decreased in CsA-treated gingival fibroblasts and tissues, but those of TSP1 and angiogenic genes were not changed. These results suggest that increased vascularity in CsA-induced gingival overgrowth tissue could result from the down-regulation of angiostatic TSP2 expression rather than the up-regulation of angiogenic factor, and that TSP2 is more important than TSP1 in the development of gingival toxicity by CsA.

Presently, there is no direct evidence as to how CsA suppresses TSP2 expression and what mediates the cellular signaling pathway during the expressional down-regulation. However, there are some clues that CsA could regulate genetic expression through the modification of transcriptional activities. Nuclear factor of activated T cells (NFAT) and NF*k*B act as transcriptional factors for some lymphocytic cytokine genes such as interleukins, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, and interferon, and

CsA–CyP complexes inhibit transcriptional activities of the factors with the alteration of calcineurin activity (32–34). Therefore, there is a possibility that TSP2 down-regulation in the CsA-treated gingival tissues and cells could result from the inhibition of calcineurin and a subsequent modification of transcriptional activity.

Recently, other angiostatic factors, brain-specific angiogenesis inhibitors (BAI1, BAI2, and BAI3), were isolated. They have a similar structure and action on angiogenesis as TSPs; they have four or five TSP-type 1 repeat domains and inhibit the bFGF-induced corneal angiogenesis *in vivo* like TSPs (35, 36). Transcripts of brain-specific angiogenesis inhibitors were detected in various tissues and cells by RT–PCR analyses unlike their nomenclatures. In the malignant glioblastoma tissue, BAI1 was prominently down-regulated and severe vascularization was observed (35). In the extracranial colorectal cancer, BAI1 expression was also inversely correlated with vascularity and distant metastasis (37). However, in our CsA-treated gingival tissues and cultured fibroblast cells, expressions of angiostatic BAI1 and BAI2 did not change. It indicates that these factors did not have a role in the development of CsA-induced gingival overgrowth, and their expressional regulations may be different from TSP2.

Taken together, CsA-induced gingival vascularity was accompanied by decreased expression of angiostatic TSP2 rather than expressional changes of other angiostatic and angiogenic genes, and it suggests that decreased expression of TSP2 in gingival tissues may play a role in the development of CsA-induced gingival overgrowth.

Acknowledgements

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References

1. Seymour RA, Jacobs DJ. Cyclosporin and the gingival tissues. *J Clin Periodontol* 1992;**19:**1–11.
2. Schreiber SL. Immunophilin-sensitive protein phosphatase action in cell signaling pathways. *Cell* 1992;**70:**365–368.
3. Mascarell L, Truffa-Bachi P. Cyclosporin A therapy differently affects immunological-relevant gene expression following immunization. *Immunol Lett* 2002;**84:** 137–143.
4. Atkinson K, Biggs J, Darveniza P, Boland J, Concannon A, Dodds A. Cyclosporin-associated central nervous system toxicity after allogeneic bone marrow transplantation. *Transplantation* 1984;**38:**34–37.
5. Bartold PM. Cyclosporine and gingival overgrowth. *J Oral Pathol* 1987;**16:**463–468.
6. Tyldesley WR, Rotter E. Gingival hyperplasia induced by cyclosporin-A. *Br Dent J* 1984;**157:**305–309.
7. Rostock MH, Fry HR, Turner JE. Severe gingival overgrowth associated with cyclosporine therapy. *J Periodontol* 1986; **57:**294–299.
8. Friskopp J, Klintmalm G. Gingival enlargement. A comparison between cyclosporine and azathioprine treated renal allograft recipients. *Swed Dent J* 1986; **10:**85–92.
9. Ayanoglou CM, Lesty C. Cyclosporin A-induced gingival overgrowth in the rat: a histological, ultrastructural and histomorphometric evaluation. *J Periodont Res* 1999;**34:**7–15.
10. Wondimu B, Reinholt FP, Modeer T. Stereologic study of cyclosporin A-induced gingival overgrowth in renal transplant patients. *Eur J Oral Sci* 1995; **103:**199–206.
11. Kataoka M, Shimizu Y, Kunikiyo K *et al.* Cyclosporin A decreases the degradation of type I collagen in rat gingival overgrowth. *J Cell Physiol* 2000;**182:**351–358.
12. Bolzani G, Della Coletta R, Martelli Junior H, Martelli Junior H, Graner E. Cyclosporin A inhibits production and activity of matrix metalloproteinases by gingival fibroblasts. *J Periodont Res* 2000; **35:**51–58.
13. Kalluri R, Sukhatme VP. Fibrosis and angiogenesis. *Curr Opin Nephrol Hypertens* 2000;**9:**413–418.
14. Ribatti D, Vacca A, Cantatore FP *et al.* An experimental study in the chick embryo chorioallantoic membrane of the anti-angiogenic activity of cyclosporine in rheumatoid arthritis versus osteoarthritis. *Inflamm Res* 2000;**49:**418–423.
15. Esposito C, Fornoni A, Cornacchia F *et al.* Cyclosporine induces different responses in human epithelial, endothelial and fibroblast cell cultures. *Kidney Int* 2000;**58:**123–130.
16. Wassef R, Cohen Z, Nordgren S, Langer B. Cyclosporine absorption in intestinal transplantation. *Transplantation* 1985;**39:** 496–499.
17. Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Peruzzi CA, Detmar M. Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the $\alpha_v\beta_3$ integrin, osteopontin, and thrombin. *Am J Pathol* 1996;**149:**293–305.
18. Senger DR, Claffey KP, Benes JE, Perruzzi CA, Sergiou AP, Detmar M. Angiogenesis promoted by vascular endothelial growth factor: regulation through $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. *Proc Natl Acad Sci USA* 1997;**94:**13612–13617.
19. Connolly DT, Heuvelman DM, Nelson R *et al.* Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest* 1989;**84:**1470–1478.
20. Detmar M. The role of VEGF and thrombospondins in skin angiogenesis. *J Dermatol Sci* 2000;**24:**S78–S84.
21. Yuan K, Jin YT, Lin MT. The detection and comparison of angiogenesis-associated factors in pyogenic granuloma by immunohistochemistry. *J Periodontol* 2000;**71:** 701–709.
22. Shihab FS, Bennett WM, Yi H, Andoh TF. Expression of vascular endothelial growth factor and its receptors Flt-1 and KDR/Flk-1 in chronic cyclosporine nephrotoxicity. *Transplantation* 2001; **72:**164–168.
23. Hernandez GL, Volpert OV, Iniguez MA *et al.* Selective inhibition of vascular endothelial growth factor-mediated angiogenesis by cyclosporin A: roles of the nuclear factor of activated T cells and cyclooxygenase 2. *J Exp Med* 2001; **193:**607–620.
24. Saito K, Mori S, Iwakura M, Sakamoto S. Immunohistochemical localization of transforming growth factor β, basic fibroblast growth factor and heparan sulphate glycosaminoglycan in gingival hyperplasia induced by nifedipine and phenytoin. *J Periodont Res* 1996;**31:**545–555.
25. Sasaki T, Maita E. Increased bFGF level in the serum of patients with phenytoin-induced gingival overgrowth. *J Clin Periodontol* 1998;**25:**42–47.
26. Tolsma SS, Volpert OV, Good DJ, Frazier WA, Polverini PJ, Bouck N. Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity. *J Cell Biol* 1993; **122:**497–511.
27. Grossfeld GD, Ginsberg DA, Stein JP *et al.* Thrombospondin-1 expression in bladder cancer: association with p53 alterations, tumor angiogenesis, and tumor progression. *J Natl Cancer Inst* 1997;**89:**219–227.
28. Tokunaga T, Oshika Y, Abe Y *et al.* Vascular endothelial growth factor

(VEGF) mRNA isoform expression pattern is correlated with liver metastasis and poor prognosis in colon cancer. *Br J Cancer* 1998;**77:**998–1002.
29. Tokunaga T, Nakamura M, Oshika Y *et al*. Alterations in tumour suppressor gene p53 correlate with inhibition of thrombospondin-1 gene expression in colon cancer cells. *Virchows Arch* 1998; **433:**415–418.
30. Oshika Y, Masuda K, Tokunaga T *et al*. Thrombospondin 2 gene expression is correlated with decreased vascularity in non-small cell lung cancer. *Clin Cancer Res* 1998;**4:**1785–1788.
31. Streit M, Riccardi L, Velasco P *et al*. Thrombospondin-2: a potent endogenous inhibitor of tumor growth and angiogenesis. *Proc Natl Acad Sci USA* 1999;**96:** 14888–14893.
32. Alzuherri H, Chang KC. Calcineurin activates NF-κB in skeletal muscle C2C12 cells. *Cell Signal* 2003;**15:**471–478.
33. Jorgensen KA, Koefoed-Nielsen PB, Karamperis N. Calcineurin phosphatase activity and immunosuppression. A review on the role of calcineurin phosphatase activity and the immunosuppressive effect of cyclosporin A and tacrolimus. *Scand J Immunol* 2003;**57:**93–98.
34. Aoki Y, Kao PN. Cyclosporin A-sensitive calcium signaling represses NFkappaB activation in human bronchial epithelial cells and enhances NFkappaB activation in Jurkat T-cells. *Biochem Biophys Res Commun* 1997;**234:**424–431.
35. Nishimori H, Shiratsuchi T, Urano T *et al*. A novel brain-specific p53-target gene, BAI1, containing thrombospondin type 1 repeats inhibits experimental angiogenesis. *Oncogene* 1997;**15:**2145–2150.
36. Koh JT, Lee ZH, Ahn KY *et al*. Characterization of mouse brain-specific angiogenesis inhibitor 1 (BAI1) and phytanoyl-CoA alpha-hydroxylase-associated protein 1, a novel BAI1-binding protein. *Brain Res Mol Brain Res* 2001;**87:**223–237.
37. Fukushima Y, Oshika Y, Tsuchida T *et al*. Brain-specific angiogenesis inhibitor 1 expression is inversely correlated with vascularity and distant metastasis of colorectal cancer. *Int J Oncol* 1998;**13:** 967–970.