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Cyclosporin A-induced Gingival Overgrowth is Closely Associated with Regulation Collagen Synthesis by the Beta Subunit of Prolyl 4-hydroxy-lase and Collagen Degradation by Testican 1- mediated Matrix Metallo-proteinase-2 Expression

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Gingival overgrowth can cause dental occlusion and seriously interfere with mastication, speech, and dental hygiene. It is observed in 25 to 81 % of renal transplant patients treated with cyclosporine A (CsA). CsA-induced gingival overgrowth (CIGO) is caused by quantitative alteration of the extracellular matrix components, particularly collagen. However, the molecular mechanisms involved in the pathogenesis of CIGO remain poorly understood, despite intense clinical and laboratory investigations. The aim of the present work is to identify differentially expressed genes closely associated with CIGO. Human gingival fibroblasts were isolated by primary explant culture of gingival tissues from five healthy subjects (HGFs) and two patients with the CIGO (CIGO-HGFs). The proliferative activity of CsA-treated HGFs and CIGO-HGFs was examined using the MTT assay. The identification of differentially expressed genes in CsA-treated CIGO-HGF was performed by differential display reverse transcriptase-polymerase chain reaction (RT-PCR) followed by DNA sequencing. CsA significantly increased the proliferation of two HGFs and two CIGO-HGFs, whereas three HGFs were not affected. Seven genes, including the beta subunit of prolyl 4-hydroxylase (P4HB) and testican 1, were upregulated by CsA in a highly proliferative CIGO-HGF. The increased P4HB and testican-1 mRNA levels were confirmed in CsA-treated CIGO-HGFs by semiquantitative RT-PCR. Furthermore, CsA increased type I collagen mRNA levels and suppressed MMP-2 mRNA levels, which are regulated by P4HB and testican-1, respectively. These results suggest that CsA may induce gingival overgrowth through the upregulation of P4HB and testican-1, resulting in the accumulation of extracellular matrix components.

Key words: cyclosporine A; gingival overgrowth; beta subunit of proline 4-hydroxylase; testican-1

Introduction

Cyclosporin A (CsA) is the most frequently used immunosuppressor for the prevention of the organ transplant rejection because of its low toxicity and potential application in the management of a variety of systemic disorders (Wysocki *et al*., 1983). Despite its considerable success as a truly selective immunosuppressant drug, CsA therapy is also associated with various adverse side effects

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such as nephropathy, hypertension, hepatotoxicity, neurotoxicity, and gingival overgrowth (Tyldesley and Rotter, 1984). Gingival overgrowth is observed between 25 to 81 % of renal transplant patients treated with CsA. The enlargement of gingival tissue can allow the accumulation of oral bacteria into the pockets followed by the marginal periodontitis and systemic infection in some cases. Therefore, the better understanding of the pathogenesis of the drug-induced gingival overgrowth is one of the important subjects in clinical periodontology.

Clinical and cellular studies associated with CsA-induced gingival overgrowth (CIGO) have demonstrated that CsA affects the gingival fibroblast proliferation (Bartold, 1989; Park *et al*., 2007) and connective tissue extracellular matrix (ECM) accumulation, resulting from the abnormal synthesis of collagen and other ECM molecules (Tipton *et al*., 1991) and the decreases of the activity of collagen degrading enzymes (Barber *et al*., 1992; Schincaglia *et al*., 1992). In addition, it has been suggested that the impaired ability of matrix degradation may play an important role in CIGO rather than the increase of the number of fibroblasts (Nares *et al*., 1996). Therefore, studies have focused on interstitial collagens (Barber *et al*., 1992; Gagliano *et al*., 2004) and matrix metalloproteinases (MMPs) as the main targets of CsA (Bolzani *et al*., 2000).

Type I collagen is the main collagen species in all layers of gingival connective tissue. Its content is determined by a dynamic balance between synthesis and degradation, and disturbances in collagen turnover may be important in the development of GO. While a stimulating effect of CsA on type I collagen synthesis has been reported (Schincaglia *et al*., 1992; Gagliano *et al*. 2004), some workers demonstrated that collagen synthesis was not affected (Bartold *et al*., 1989) or inhibited by CsA (Barber *et al*., 1992). Analysis of immunohistological preparations in cases of GO demonstrated that type IV collagen was present in significantly higher amounts in the CsA-treated gingiva than in healthy gingiva, but type III collagen was not significantly greater (Bonnaure-Mallet *et al*., 1995).

MMP family of zinc-dependent endopeptidases is responsible for the remodeling of ECM in both physiological and pathological processes (Cotrim *et al*., 2002). Although MMPs are broadly divided into interstitial collagenases, gelatinases, stromelysins, and membrane-type MMPs, each group can degrade essentially the ECM components. Evidence has been shown that CsA inhibits significantly the activity of MMP-1, MMP-2 and MMP-3 (Bolzani *et al*., 2000; Yamada *et al*., 2000; Cotrim *et al*., 2002; Hyland *et al*., 2003; Gagliano *et al*., 2004; Kim *et al*., 2008), thereby contributing to the extracellular matrix accumulation found in CIGO. However, the molecular mechanisms involved in the pathogenesis of CIGO remain poorly understood despite intense clinical and laboratory investigation. The aim of the present work is to explore the molecular mechanisms of CIGO by identifying the differentially expressed genes closely associated with CIGO.

Materials and Methods

Chemicals

Cyclosporin A (CsA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), isopropanol and chloroform were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), antibiotic-antimycotic mixture (10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B) and trypsin-EDTA solution (0.25 % trypsin, 1 mM EDTA) were purchased from Invitrogen Corporation (Grand Island, NY, USA).

Cell cultures

Human gingival fibroblasts were obtained using primary explant culture of gingival tissues from 5 healthy subjects without evidences of inflammation, hyperplasia or the history of taking drugs associated with gingival overgrowth, and from 2 patients with the CsA-induced gingival overgrowth, respectively. The donors were from 27 to 65 years old. Informed consent was obtained from each subject. The protocol for the study was approved by Institutional Review Board of Yonsei University College of Dentistry, Seoul, Korea. Gingival biopsies were washed with sterile PBS and plated in 25 cm^2 culture flasks, and incubated with DMEM supplemented with 20 % heat-inactivated FBS and 2 % antibiotic-antimycotic mixture at 37 ^{o}C in a humidified atmosphere of 5 % CO_2. When reaching confluence, the cells were trypsinized with 0.25 % trypsin containing 1 mM EDTA. All experiments were performed with cells between the third and sixth passages.

Cell proliferation assay

Human gingival fibroblasts from healthy subjects (HGFs) or patients with CIGO (CIGO- HGFs) were seeded at 1×10^3 cells/well in DMEM containing 10 % FBS in 96-well plates. 24 h later, the medium was replaced to fresh 2 % FBS-DMEM containing the various concentrations of CsA (0, 0.1, 1.0, 10, 100 ng/ml), and the cells were cultured for 3 days and 5 days. CsA (1 mg/ml) was dissolved in DMSO and diluted with 2 % FBS-DMEM. Control cells received 0.01 % DMSO alone. When cells reached confluence, a MTT solution (final concentration: 0.5 mg/ml) was added to each well and incubated for 4 h at 37 ^{o}C. The MTT solution was removed and the remaining formazan products were dissolved by 100 μl of DMSO. Absorbance was measured at 570 nm using spectrophotometric microplate reader (Bio-Rad, Hercules, CA, USA).

Differential display reverse transcriptase-polymerase chain reaction (DDRT-PCR)

Total cellular RNA was isolated from CIGO-HGFs treated with CsA (0, 0.1, 1.0, 10 ng/ml) using TRIzol reagent (Life technologies, Austria). DDRT-PCR was performed using the RNAimage kit (GenHunter Corp., Nashville, TN, USA). The DNase I-treated total RNA pools (200 ng per each group) were subjected to reverse transcription in reverse transcriptase buffer (25 mM Tris-HCl, pH 8.3, 37.6 mM KCl, 1.5 mg $MgCl_2$ and 5 mM DTT) with 5 unit/μl of MMLV-reverse transcriptase, 20 μM dNTP mix and 0.2 μM of guanosine-anchored oligo(dT) primer (HT_{11}-G). The cDNA samples were used for PCR in dilution of 1:10. PCR (20 μl) was performed in PCR buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001 % gelatin) containing 2 μM dNTP, 0.2 μM of HT_{11}-G, 0.2 μM of arbitrary primer (from H-AP1 to H-AP10), 0.2 μl of α-[^{33}P]dATP (2000 Ci/mmole), and 0.05 unit/μl of AmpliTaq DNA Polymerase (Perkin Elmer Inc., Wellesley, MA, USA). The thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Australia) was programmed as follows: 40 cycles at 94 °C for 30 sec, 40 °C for 2 min, and 72 °C for 30 sec, and terminated with a final extension at 72 °C for 5 min. Radiolabeled PCR products were separated on 6 % denaturing polyacrylamide gel and the bands visualized by autoradiography.

Cloning and DNA sequencing

The differentially expressed cDNA fragments identified on the autoradiogram were excised from the gel, eluted by boiling in water and reamplified by PCR with the same set of primers and conditions used in DD-PCR. The reamplified PCR products were cloned in PCR-TRAP vector using PCR-TRAP cloning system (GenHunter Corp.) according to the manufacturer's instructions. Sequencing of cloned cDNAs was performed at Takara Korea Biomedical Inc. (Suwon, Korea) and the sequence alignment was performed in GenBank of National Center for Biotechnology Information (NCBI) using standard nucleotide-nucleotide BLAST (blastn) program (http://www.ncbi.nlm.nih.gov/BLAST/) and all EMBL libraries using Fasta3 program (http://www.ebi.ac.uk/fasta3/).

RT-PCR

Semi-quantitative RT-PCR was performed to confirm the results from DDRT-PCR and to investigate mRNA expression levels of type I collagen and metalloproteinase-2 (MMP-2) in CIGO-HGFs treated by CsA. First-strand cDNA was synthesized with 1 μg of total RNAs and 1 μM of oligo-dT_{15} primer using MMLV Reverse Transcriptase (Promega, Medison, CA, USA). Using the recombinant Taq DNA polymerase kit (Takara, Shiga, Japan), PCR was performed with 0.5 μg of first-strand cDNA and 20 pmole of primers (Table 1). PCR reaction consisted of initial denaturation at 94 °C for 3 min, 30 cycles at 94 °C for 40 sec, 52 °C for 40 sec (61 °C for type I collagen), and 72 °C for 1 min, and final extension at 72 °C for 10 min. The amplified PCR products were electrophoresed on a 1.5 %-2 % agarose gel and visualized by staining the gel with ethidium bromide. The mRNA expression level of each gene was normalized with that of glyceraldehyde-3-phsophate dehydrogenase (GAPDH).

Statistical Analysis

Statistical analysis of the data was performed using the unpaired Student's *t* test. The *p* value less than 0.05 was considered to be statistically significant.

Results

Among 5 HGFs isolated from 5 healthy subjects, the proliferation of 2 HGFs was increased meaningly by CsA treatment for 3 and 5days (Fig. 1A-a), whereas that of 3 HGFs were not changed (Fig.1A-b). In contrast, all CIGO-HGFs from 2 patients with gingival overgrowth showed significantly increased proliferation when cultured with CsA for 3 and 5 days (Fig. 1B).

To identify the differentially expressed genes associated with CsA-induced proliferation in CIGO-HGFs, DDRT-PCR was performed. As described in Table 2, seven genes were upregulated by CsA treatment. Among them, the increased mRNA expression levels of β subunit of prolyl 4-hydroxylase (P4HB; Fig. 2A) and testican-1 (Fig. 2B) were confirmed in CsA-induced highly proliferated CIGO-HGFs

Table 1. Primers.

Target genes	Sequences	Product size(bp)
Testican 1	Forward 5'-TGTGTGACCCAGGACTACCA-3' Reverse 5'-ACTTGTTGAACATCCAGCCC-3'	478
P4HB	Forward 5'-GGAGATGACCAAGTACAAGC-3' Reverse 5'-GGCTTTGCGTATTACAGTTC-3'	573
MMP-2	Forward 5'-GTCGCCCATCATCAAGTTC-3' Reverse 5'-CTCCCAAGGTCCATAGCTCA-3'	557
Type I collagen	Forward 5'-GGCGGCCAGGGCTCCGAC-3' Reverse 5'-CCACGGGGTCTGGTCCTTAA-3'	347
GAPDH	Forward 5'-GTCAGTGGTGGACCTGACCT-3' Reverse 5'-AGGGGTCTACATGGCAACTG-3'	420

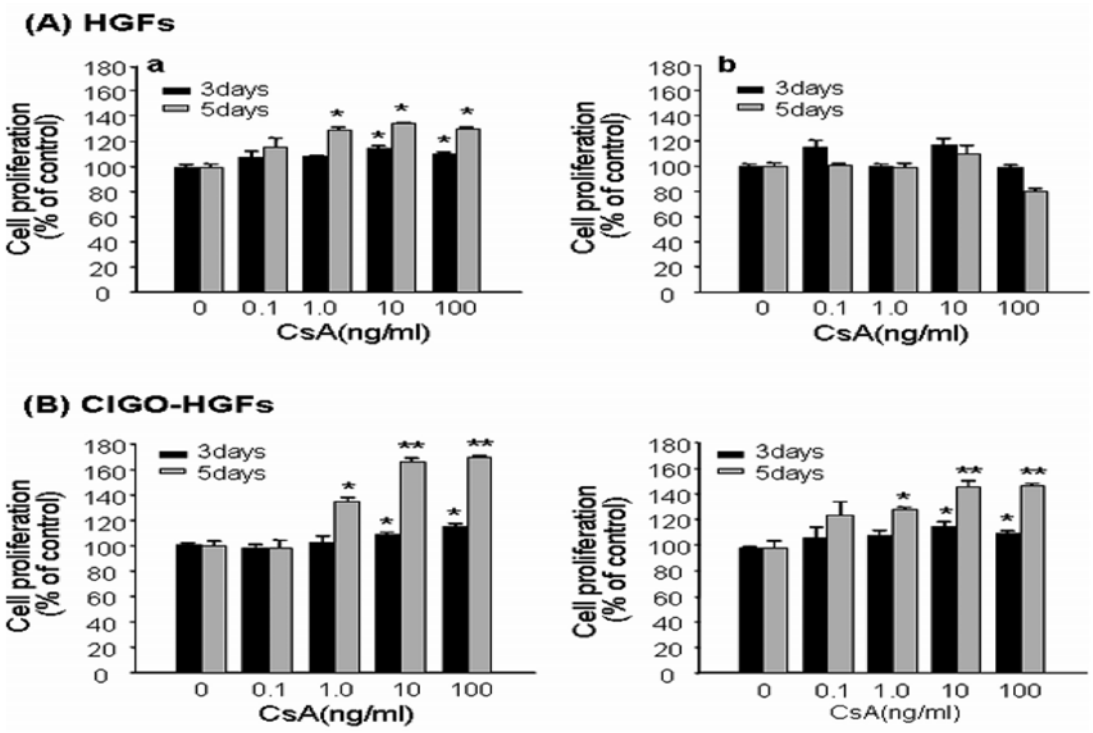

Fig. 1. The effect of CsA on the proliferation of human gingival fibroblasts. Five HGFs and two CIGO-HGFs were stimulated with CsA at the indicated doses for 3 and 5 days, respectively. Cell proliferation was measured by MTT assay. Graph A shows the representatives of two HGFs indicating the increased proliferation by CsA treatment (a), and three HGFs which were not affected by CsA treatment (b). The graph B represents CsA-induced proliferation of two CIGO-HGFs. The results are expressed as the mean ± SE of triplicate assays. $*P < 0.05$, $**P < 0.01$.

Table 2. Genes upregulated by CsA treatment in CIGO-HGFs.

Clone No.	Acession No.	Definition	Homology
3	NM_000918.2	Homo sapiens procollagen-proline, oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55) (P4HB), mRNA	100
15	AF231124.1	Homo sapiens testican 1 mRNA, complete cds	96
17	NM_000366.3	Homo sapiens tropomyosin 1 (alpha) (TPM1), mRNA	100
24	AAN37426.1	NADH dehydrogenase (ubiquinone) (EC1.6.5.3) chain 2-human mitochondrion, partial (84 %)	94
33	M94314.1	Homo sapiens ribosomal protein L30, mRNA, complete cds	95
34	BC000690.1	Homo sapiens ribosomal protein L24, mRNA (cDNA clone MGC:2240 IMAGE:3349215), complete cds	100
36	BC040354.1	Homo sapiens, similar to caldesmon 1, clone MGC:21352 IMAGE:4753285, mRNA, complete cds	100

by semiquantitive RT-PCR. Furthermore, mRNA expression levels of type I collagen (Fig. 3A) and MMP-2 (Fig. 3B) were increased in CsA-induced highly proliferated CIGO-HGFs.

Discussion

Gingival overgrowth (GO), seriously interfering with occlusion, mastication, speech and dental hygiene, is

observed in a significant number of cases treated with immunosuppressants (CsA, etc), antiepileptics (phenytoin, etc) and calcium channel antagonists (nifedipine, etc). It has been known that the drugs resulting in GO directly or indirectly influence both the growth and function of the gingival fibroblasts, which play an important role in ECM turnover through the production of both matrix macromolecules and matrix-degrading enzymes such as MMPs (Nares *et al*., 1996; Atilla and Kutukculer, 1998; Swarga *et al*., 2001). Up to date, various cell culture studies have focused on connective tissue extracellular matrix accumulation to elucidate the pathogenesis of gingival

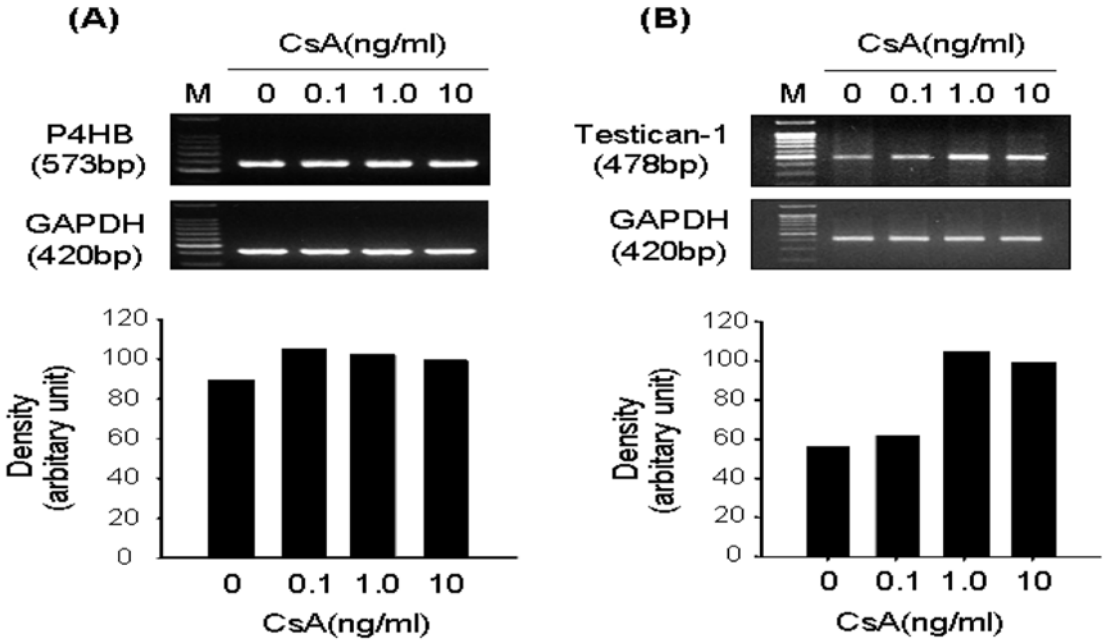

Fig. 2. mRNA expression of P4HB and testican 1 in CsA-stimulated CIGO-HGFs. Semi-quantitative RT-PCR was performed to investigate mRNA expression levels of P4HB and testican I as described in Materials and Methods.

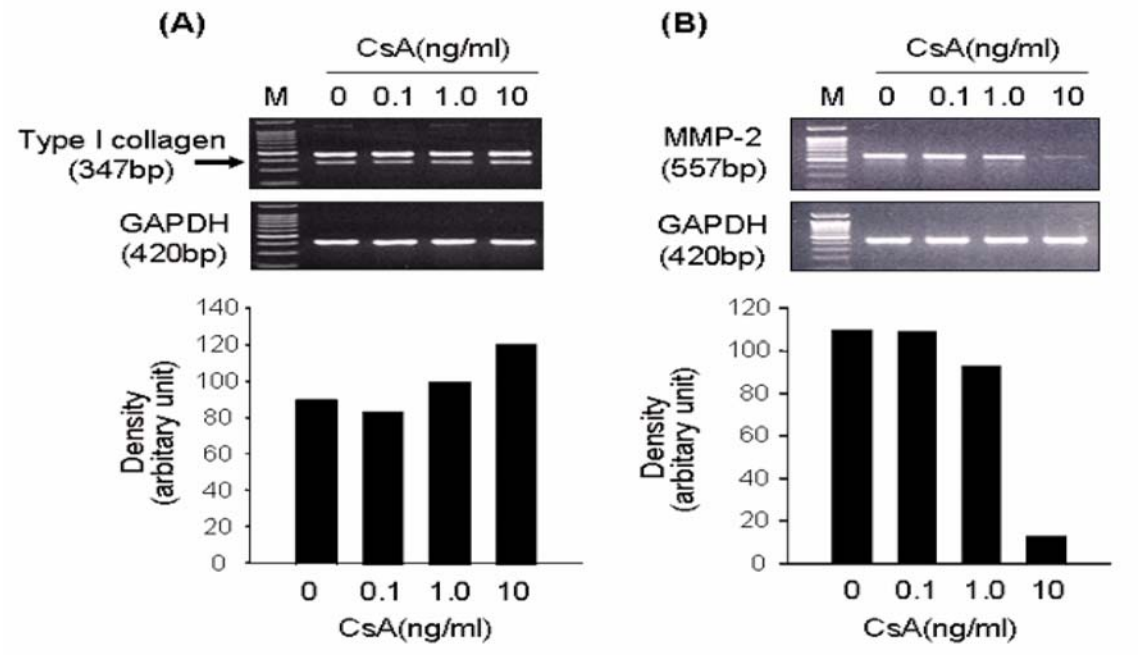

Fig. 3. mRNA expression of type I collagen and MMP-2 in CsA-stimulated CIGO-HGFs. Semi-quantitative RT-PCR was performed to investigate mRNA expression levels of P4HB and type I collagen as described in Materials and Methods.

overgrowth.

In this study, CsA treatment increased the proliferation of two HGFs and all CIGO-HGFs, but that of three HGFs was not changed by CsA, when cultured between 0.1 ng/ml and 100 ng/ml of CsA for 3 and 5 days. Two CIGO-HGFs, rather than HGFs, proliferated more significantly by CsA stimulation. These results suggest that the priming of the gingival tissue with cyclosporin in human can lead to the increased susceptibility of the fibroblasts on CsA *in vitro.*

To investigate the molecular mechanisms by which CsA increases the proliferation of human gingival fibroblasts and extracellular matrix accumulation, the differentially expressed genes following CsA treatment were identified in a highly proliferating CIGO-HGF by CsA stimulation using DDRT-PCR. Among seven genes upregulated by CsA treatment, P4HB is a multifunctional protein regarded as the protein disulfide isomerase (PDI) (Koivu *et al*., 1987). PDI catalyzes the formation of disulfide bonds in type I and II procollagens (Koivu and Myllylä, 1987) and prolyl 4-hydroxylase (Koivu and Myllylä, 1986), a key enzyme of collagen synthesis. The function of PDI as a component of prolyl 4-hydroxylase appears to be to maintain the catalytic subunits in a soluble form rather than directly participating in catalysis, and thereby participates during proline hydroxylation (John *et al*., 1993). PDI also acts as a molecular chaperone assisting the folding of polypeptides (Cai *et al*., 1994). More recently, the association of PDI with type I procollagen has been proposed to lead to endoplasmic reticulum retention of type I procollagen, which is composed of two proα1(I) chains and one proα2(I) chain and intracellularly degraded immediately after its synthesis, in corneal endothelial cells (Ko *et al*., 2004). Furthermore, it has been reported that the proyl 4-hydroxylase inhibitor is more effective for the inhibition of proliferation than for inhibition of collagen synthesis of rat hepatic stellate cells (Aoyagi *et al*., 2002). Therefore, our data demonstrate that the overexpression of PDI mRNA by CsA in CIGO-HGFs may increase cell proliferation while producing collagen, by stabilizing prolyl 4-hydroxylase and delaying the intracellular degradation of type I procollagen. In addition, these results can provide the molecular basis of other *in vitro* studies that CsA causes a significant increase in the level of type I procollagen and cell proliferation (Bolzani *et al*., 2000).

Testican-1 is a highly conserved chimeric proteoglycan carrying both chondroitin sulfate and heparan sulfate chains (Alliei *et al*., 1993). Testican-1 has been shown to inhibit the activation of pro-MMP-2 by either MT1-MMP or MT3-MMP (Nakada *et al*., 2001) as well as the activity of lysosomal enzyme cathepsin L (Bocock *et al*., 2003), regulating the degradation of extracellular matrices (Nakada *et al*., 2003). Recent studies have reported that the reduced activity of cathepsin-L (Yamada et al., 2000) and matrix metalloproteinases (Bozani *et al*., 2000; Cotrim *et al*., 2002; Hyland *et al*., 2003; Gangliano *et al*., 2004) by CsA, resulting in the inhibition of protein degradation in gingival connective tissues, plays important roles in the pathogenesis of CIGO. Our result suggests that the suppression of MMP-2 expression in CIGO can be due to the overexpression of testican-1 by CsA. Furthermore, alterations of type I collagen and MMP-2 expression following the upregulation of P4HB and testican-1 mRNA levels were assessed in CIGO-HGF cells treated with CsA by RT-PCR. We confirmed that CsA increased type I collagen mRNA levels and suppressed MMP-2 mRNA levels.

In conclusion, CsA may induce the gingival overgrowth through the upregulation of P4HB and testican-1, resulting in the increase of collagen synthesis and the accumulation of extracellular matrix components by inhibiting MMP-2 activity, in patients receiving CsA therapy. This is the first report demonstrating that the accumulation of collagen in CIGO regulates by P4HB and testican-1.

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