J Periodont Res 2009; 44: 338–347

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2008.01126.x

Cyclosporine A inhibits the expression of membrane type-I matrix metalloproteinase in gingiva

H-C. Chiu[1,2], Y-T. Lu[2], Y-T. Chin[2,3], H-P. Tu[2], C-Y. Chiang[2], C-H. Gau[4], S. Nieh[5], E. Fu[2]

[1]Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan, China, [2]Department of Periodontology, School of Dentistry, National Defense Medical Center and Tri-Service General Hospital, Taipei, Taiwan, China, [3]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan, China, [4]Department of Nursing, Kang-Ning Junior College of Medical Care and Management, Taipei, Taiwan, China and [5]Department of Pathology, National Defense Medical Center and Tri-Service General Hospital, Taipei, Taiwan, China

Chiu H-C, Lu Y-T, Chin Y-T, Tu H-P, Chiang C-Y, Gau C-H, Nieh S, Fu E. Cyclosporine A inhibits the expression of membrane type-I matrix metalloproteinase in gingiva. J Periodont Res 2009; 44: 338–347. © 2009 The Authors. Journal compilation © 2009 Blackwell Munksgaard

Background and Objective: Membrane type-I matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase-2 (TIMP-2) regulate the activation of MMP-2; however, their roles in the activation of MMP-2 in gingiva during treatment with cyclosporine A are still unknown. Therefore, the expressions of membrane type-I MMP and TIMP-2, as well as MMP-2, in gingivae upon treatment with cyclosporine A were examined *in vivo* and *in vitro*.

Material and Methods: Thirty-four rats were divided into two groups after edentulous ridges were established. The experimental group received 30 mg/kg/d of cyclosporine A and the control group received vehicle. At the end of the experimental period, the rats were killed, the gingivae were obtained and the expression of mRNA and protein of membrane type-I MMP, TIMP-2 and MMP-2 in gingiva were examined using real-time polymerase chain reaction and immunohistochemistry. In human gingival fibroblasts, the activity of MMP-2 and the expression of MMP-2, membrane type-I MMP and TIMP-2 mRNAs were examined (using zymography and reverse transcription–polymerase chain reaction, respectively) after treatment with cyclosporine A.

Results: In gingivae of rats, cyclosporine A significantly decreased the expression of mRNA and protein of membrane type-I MMP, but not of TIMP-2. The expression of MMP-2 mRNA was unaffected but the expression of MMP-2 protein showed a significant decrease upon treatment with cyclosporine A. In fibroblast culture medium, the presence of cyclosporine A induced a decrease in MMP-2 activity in a dose-dependent manner. The expression of MMP-2, membrane type-I MMP and TIMP-2 mRNAs in fibroblasts was not significantly affected by cyclosporine A; however, in fibroblasts the ratio of mRNA expression of membrane type-I MMP to that of TIMP-2 decreased as the cyclosporine A dose was increased.

Conclusion: Cyclosporine A inhibits the expression of membrane type-I MMP in gingiva and it may further reduce the activation of MMP-2.

Dr Earl Fu, Department of Periodontology, School of Dentistry, National Defense Medical Center, PO Box 90048-507, Taipei, Taiwan, China
Tel: +886 2 87927150
Fax: +886 2 87927145
e-mail: dentalab@tpts5.seed.net.tw

Key words: cyclosporine; adverse effects; gingiva; membrane type-I matrix metalloproteinase; matrix metalloproteinase-2

Accepted for publication May 7, 2008

Gingival overgrowth induced by cyclosporine A, an immunosuppressant, is characterized by epithelial hyperplasia, interstitial fibrosis and focal inflammatory cell infiltration (1). Although complex interactions among mediators of tissue remodeling and inflammation may be involved in gingival overgrowth, the exact mechanism

is not fully understood. Cyclosporine A affects fibroblast proliferation, promotes abnormal accumulation of extracellular matrix components in the gingival lamina propria and appears to affect the catabolic enzymes of the extracellular matrix (2–6).

The matrix metalloproteinases (MMPs) are a family of structurally related proteins that degrade most, if not all, components of the extracellular matrix and basal membranes in a zinc-dependent manner at physiological pH (7,8). They have been implicated in extracellular matrix remodeling in embryonic development, inflammation, tumor invasion, metastasis and fibrosis (9). Matrix metalloproteinase-2 plays a pivotal role in remodeling basement membranes via pericellular and cell-attachment proteins (10–12). Matrix metalloproteinase-2 is secreted from cells as an inactive zymogen, pro-MMP-2. Activation occurs via a membrane-linked process that involves membrane type-1 MMP (MMP-14 metalloproteinase) (13) and tissue inhibitor of metalloproteinase-2 (TIMP-2) (14,15). Membrane type-I MMP, like the other five membrane type MMPs, differs from other MMPs in that it has a transmembrane domain that localizes the enzyme to the plasma membrane in addition to the three basic domains that characterize all other MMPs (13). A complex consisting of membrane type-I MMP and TIMP-2 serves as a cell-surface receptor for pro-MMP-2. The cleavage of pro-MMP-2 (68 kDa) to activate MMP-2 (62 kDa) is accomplished by a TIMP-2-free neighboring membrane type-I MMP molecule. Free TIMP-2, on the other hand, inhibits this cleavage reaction/activation. Thus, the ratio of membrane type-I MMP to TIMP-2 is critical for MMP-2 activation (15–18).

Cyclosporine A has been demonstrated to decrease MMP-2 activity (19–21) and increases deposition of type-IV collagens (22,23). Some studies have also shown that periodontal pathogens activate MMP-2 by affecting the activities of membrane type-I MMP and TIMP-2 (24,25). Whether such regulation occurs in cyclosporine A-induced gingival overgrowth is unknown. We therefore studied the *in vivo* and *in vitro* expression of gingival membrane type-I MMP, TIMP-2 and MMP-2 after treatment with cyclosporine A to elucidate their roles in the induction of gingival overgrowth.

Material and methods

Animal study

Maxillary edentulous ridges were established in 34 male 3-wk-old Sprague Dawley rats (120–150 g), as described previously. In brief, all right maxillary molars were extracted from rats under ketamine anesthesia. After a 2-wk healing period, the rats were randomly assigned to cyclosporine A and control groups by simple random sampling. Animals in the cyclosporine A group received 30 mg/kg/d of cyclosporine A (Sandimmun; Sandoz, Basel, Switzerland) by gastric feeding for 4 wk and the rats in the control group received mineral oil alone. At the end of the fourth week, all rats were killed by carbon dioxide inhalation. The gingival and surrounding mucosae of the edentulous ridge were immediately frozen in liquid nitrogen and stored at −80°C. Three specimens from each group were prepared for real-time polymerase chain reaction (PCR) and 14 specimens (seven per group) were fixed in 4% paraformaldehyde. After dehydration and embedding, the gingivae were sectioned buccopalatally into 4-μm-thick sections and stained for immunohistochemistry analyses.

Real-time PCR

Total RNA was extracted from homogenized gingival tissue and then reverse transcribed. The PCR primers used were as follows: membrane type-I MMP, sense (5′-GAACTTCGTGTTGCCTGATG-3′) and antisense (5′-AGAGGTGGTTCTGGGTTGAG-3′), with an expected product of 123 bp (accession no.: NM_031056.1); TIMP-2, sense (5′-CAGGGAAGGCGGAAGGA-3′) and antisense (5′-CCAGGGCACAATAAAGTCACAGA-3′), with an expected product of 85 bp (accession no.: NM_021989.2); MMP-2, Rat MMP2 Gene Expression System (Applied Biosystems, Foster City, CA, USA); and β-actin, Rat β-actin Gene Expression System (Applied Biosystems). The ABI Real-time PCR system (ABI 7000® Prism Sequence Detection System; Applied Biosystems) was used, according to the manufacturer's instructions, to confirm the gene expression of membrane type-I MMP, TIMP-2 and MMP-2. In brief, the desired probes and primers for rat membrane type-I MMP, TIMP-2, MMP-2 and β-actin were selected from the TaqMan Assay-on-Demand gene expressions available from Applied Biosystems. TaqMan PCR was conducted in triplicate with 50-μL reaction volumes of 1 × PCR buffer A, 2.5 mM $MgCl_2$, 0.4 μM each primer, 200 μM each dNTP, 100 nM probe and 0.025 U/μL of Taq Gold. For each experiment, a large master mix of the above components was made and aliquoted into each optical reaction tube. Each primer/probe set (5–10 μL) was then added and PCR was conducted using the following cycle parameters: 1 cycle at 95°C for 12 min, followed by 40 cycles at 95°C for 20 s and 60°C for 1 min. Data analyses were carried out using sequence detection software that calculates the threshold cycle (C_t) for each reaction. The reaction was used to quantify the amount of starting template in the reaction. A difference in C_t values (ΔC_t) was calculated for the expression of membrane type-I MMP, TIMP-2 and MMP-2 by taking triplicate C_t values from three reactions and subtracting the mean C_t of the triplicates for the control gene, β-actin, for each cDNA sample at the same concentration. Relative gene expression for membrane type-I MMP, TIMP-2 and MMP-2 in cyclosporine A-treated or untreated individuals was calculated as $= 2^{-\Delta Ct}$ (ΔC_t = CT vegf−CT β-actin) (26).

Immunohistochemistry

Immunohistochemistry was used to evaluate the expression of MMP-2, membrane type-I MMP and TIMP-2 protein in gingival tissue. After fixation in acetone, tissue sections were incubated in distilled water containing 0.1% hydrogen peroxide to quench endogenous peroxidase activity. The tissue

sections were then incubated for 2 h with unconjugated primary polyclonal antibodies against MMP-2 (mouse IgG) (Chemicon International Inc., Temecula, CA, USA), membrane type-I MMP (rabbit IgG) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TIMP-2 (rabbit IgG) (Santa Cruz Biotechnology), and then incubated with biotinylated secondary antibody, streptavidin-conjugated horseradish peroxidase complexes and 3-amino-9-ethyl carbazole solution for 4, 20 and 20 min, respectively. Between incubations, the sections or fibroblasts were washed with sterile phosphate-buffered saline. The specimens were then washed in distilled water and counterstained with hematoxylin. The tissue sections were dehydrated and mounted for microscopic observation. Positively stained MMP-2, membrane type-I MMP and TIMP-2 cells were detected using a microscope. The percentage of cells staining positive were recorded after counting the total cells in 0.0324 mm^2 of gingival connective tissue, as described in our previous study (27).

Culture of human gingival fibroblasts

Human gingival fibroblasts were obtained as described previously (28). In brief, the gingival specimens were immersed for 2 d at 4°C in Leibovitz L-15 medium (Sigma-Aldrich Inc., St Louis, MO, USA) containing 2 mg/mL of dispase II (Roche Diagnostics, Indianapolis, IN, USA) and 10% fetal bovine serum. After separation from the outer epithelial layer, the connective tissue was minced and digested for 24 h in medium containing 10% fetal bovine serum and 2 mg/mL of collagenase (Sigma-Aldrich Inc.). The tissue was then placed in flasks containing 10% fetal bovine serum in Dulbecco's minimal essential medium/F-12 media to enable the cells to migrate from the explants. Fibroblasts were used for experiments after they had undergone four or more passages. Confluent fibroblasts were starved in serum-free medium for 2 d before being used in the experiments. The gelatinolytic activity of MMP-2 in supernatants of fibroblast cultures treated with or without cyclosporine A for 3 d was determined using zymography. The expression of MMP-2, membrane type-I MMP, TIMP-2 and glyceraldehyde-3-phosphate dehydrogenase mRNAs in fibroblasts was determined using reverse transcription–polymerase chain reactions (RT-PCR).

Cell viability analysis

Human gingival fibroblasts were placed in the wells of 96-well multiplates containing Dulbecco's minimal essential medium/F12 medium and 10% fetal bovine serum, and cultured until confluent. Then, the cells were washed once with phosphate-buffered saline and the medium was replaced with Dulbecco's minimal essential medium/F12 medium containing 1% fetal bovine serum to starvation for 48 h. Before and after cyclosporine A treatment (0, 50, 100, 500, 1000 or 5000 ng/mL in 50% dimethylsulfoxide) for 72 h, we tested cell viability using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay (CellTiter 96_AQueous One Solution; Promega, Madison, WI, USA) according to the manufacturer's protocol. The effect of cyclosporine A on the viability of gingival fibroblasts was compared before and after cyclosporine A treatment, as modified from a previous study (29).

Gelatin zymography

The release of MMP-2 from human gingival fibroblasts was evaluated using gelatin zymography. The proteins in the medium in which the fibroblasts were incubated were separated under nonreducing conditions using a 10% sodium dodecyl sulfate–polyacrylamide gel containing 0.1% gelatin. Equal amounts of protein, measured using the BCA™ protein assay (Pierce, Rockford, IL, USA), were loaded into each lane of the gel. After electrophoresis, the gel was shaken gently in renaturing buffer (2.5% Triton X-100) at room temperature (23 to 25°C) for 30 min to remove sodium dodecyl sulfate and then incubated in developing buffer containing 1 mM ZnCl, 5 mM $CaCl_2.H_2O$ and 2 M Tris–HCl (pH 8.8) at 37°C for 2 d. The gel was stained with 2.5% Coomassie Brilliant Blue in 30% methanol and 10% acetic acid. The latent and active forms of MMP-2 were detected as 72- and 62-kDa bands, respectively. The gel images were scanned directly (Transilluminator/SPOT; Diagnostic Instruments, Sterling Heights, MI, USA).

RT-PCR

Total RNA was extracted from homogenized gingival fibroblasts and then reverse transcribed. The PCR conditions were as follows: an initial denaturation at 94°C for 2 min 30 s followed by 30 or 40 cycles at 94°C for 30 s, an appropriate annealing temperature (58–60°C) for 30 s and then 72°C for 55 s. The PCR primers were: membrane type-I MMP, sense (5′-CATCGCTGCCATGCAGAAGT-3′) and antisense (5′-GTCATCATCGGGCAGCAC-3′), with an expected product of 633 bp (24); TIMP-2, sense (5′-GCGCTCGGCCTCCTGCTG-3′) and antisense (5′-CTTGATGCAGGCGAAGAACTTG-3′), with an expected product of 506 bp (30); MMP-2, sense (5′-CCACGTGACAAGCCCATGGGGCCCC-3′) and antisense (5′-GCAGCCTAGCCAGTCGGATTTGATG-3′), with an expected product of 480 bp (31); and glyceraldehyde-3-phosphate dehydrogenase, sense (5′-AGCCGCATCTTCTTTTGCGTC-3′) and antisense (5′-TCATATTTGGCAGGTTTTTCT-3′), with an expected product of 816 bp (32). Amplified RT-PCR products were analyzed using 1% agarose gels and visualized using ethidium bromide staining and a camera system (Transilluminator/SPOT; Diagnostic Instruments). The gel images were scanned directly (ONE-Dscan 1-D Gel Analysis Software; Scanalytic Inc., Fairfax, VA, USA) and the relative densities were determined as the ratio of sample signal intensity to the intensity of the glyceraldehyde-3-phosphate dehydrogenase band.

Statistical analysis

The Student's *t*-test was used to evaluate group differences in the gingival

expression of membrane type-I MMP, TIMP-2 and MMP-2 mRNAs (relative density) and the number and distribution of gingival stoma cells that were positively stained by immunohistochemistry. A one-way analysis of variance and Duncan's test for *post hoc* analysis were used to evaluate the effect of cyclosporine A dose on the *in vitro* expression of mRNA and the gelatinolytic activity of MMP-2 in gingival fibroblast cultures. Stepwise regression analysis was also used to determine the effect of cyclosporine A dose on *in vitro* gelatinolytic activity. A p-value of < 0.05 was considered significant.

Results

The mRNA expression of membrane type-I MMP in edentulous gingivae was significantly lower in cyclosporine A-treated rats than in control rats, but expression of TIMP-2 and MMP-2 did not differ significantly between groups, according to the real-time PCR results (Fig. 1). Cyclosporine A-treated rats had fewer positively stained membrane type-I MMP and MMP-2 gingival cells than control rats, irrespective of the number or distribution of cells (Fig. 2). However, the number of positively stained TIMP-2 gingival cells in the two groups was similar.

The viability test showed that the proportion of viable human gingival fibroblasts was similar after culture in plates with or without cyclosporine A for 0 and 72 h (Fig. 3). The results of zymography showed that cyclosporine A treatment induced a dose-dependent decrease in MMP-2 activity in the culture medium of fibroblast cultures ($p = 0.017$ and $R = 0.673$) (Fig. 4). The results of RT-PCR analysis showed that cyclosporine A did not significantly affect the expression of membrane type-I MMP, TIMP-2 and MMP-2 mRNAs in fibroblasts (Fig. 5A–C), However, the mRNA expression ratio of membrane type-I MMP to TIMP-2 decreased dose-dependently ($R = 0.654$, $p = 0.025$) with increasing dose (from 500 to 5000 ng/mL) of cyclosporine A (the ratio differed significantly between the 500 and 5000 ng/mL treatments) (Fig. 5D).

Discussion

Cyclosporine A induces gingival overgrowth, as observed in humans and animals (1,4,33); however, the underlying mechanism of this is still unclear. Recent studies have investigated the roles of various factors in cyclosporine A-induced gingival overgrowth, including those of growth factors, such as transforming growth factor, platelet-derived growth factor and vascular endothelial growth factor (27,34,35), and those of pro-inflammatory cytokines, such as interleukin 1β, tumor necrosis factor-α and interleukin-6 (36–38). Cyclosporine A also has catabolic effects in that it decreases collagenase activity in cultured fibroblasts and immune cells (39,40). Matrix metalloproteinases that have collagenase and gelatinase activities are secreted into the extracellular space as zymogens and are activated by proteolytic cleavage within the matrix environment. Their activity is closely regulated, but under pathological conditions an imbalance between the active and inactive forms of MMPs may result in excessive extracellular matrix accumulation or degradation (41). It is usually accepted that the balance between activated MMPs and TIMPs controls the extent of extracellular matrix remodeling and that tissue degradation is caused by disruption of this balance in favor of proteinases (42).

Because cyclosporine A significantly inhibits the production of MMP-1 and MMP-3 in overgrown gingivae of rats and in human gingival fibroblast cultures, it has been suggested that the inhibitory effects of cyclosporine A may contribute to the accumulation of extracellular matrix components in induced gingival overgrowth (19). A recent study revealed that cyclosporine A suppresses expression of MMP-1 and TIMP-1 mRNAs in human gingival fibroblast cultures in a time-dependent manner (43). Another study showed that cyclosporine A inhibits MMP-1 expression in gingival fibroblast cultures at both mRNA and protein levels in a dose-dependent and time-dependent manner (44). However, in the aforementioned study, cyclosporine A inhibited TIMP-1 mRNA, but not protein, expression. Long-term exposure to cyclosporine A (five to eight passages) did not impair the accumulation of TIMP-1 mRNA in human gingival fibroblasts (45). Another author proposed that low TIMP-1 levels are an important factor in the pathogenesis of cyclosporine A-induced gingival overgrowth because cyclosporine A did not have a

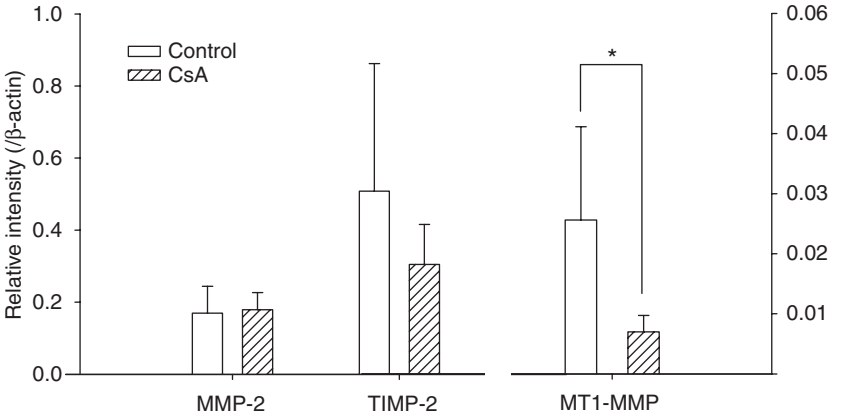

Fig. 1. Analysis, using real-time polymerase chain reaction, of the expression of matrix metalloproteinase-2 (MMP-2), membrane type-I MMP and tissue inhibitor of metalloproteinase-2 (TIMP-2) mRNAs in the gingivae of rats. The expression levels, relative to that of β-actin, were compared between gingival tissues of control and cyclosporine A-treated rats ($n = 3$ per group). Data are expressed as means and standard deviations. *Significant difference when $p < 0.05$. CsA, cyclosporine A; MT1-MMP, membrane type-I matrix metalloproteinase.

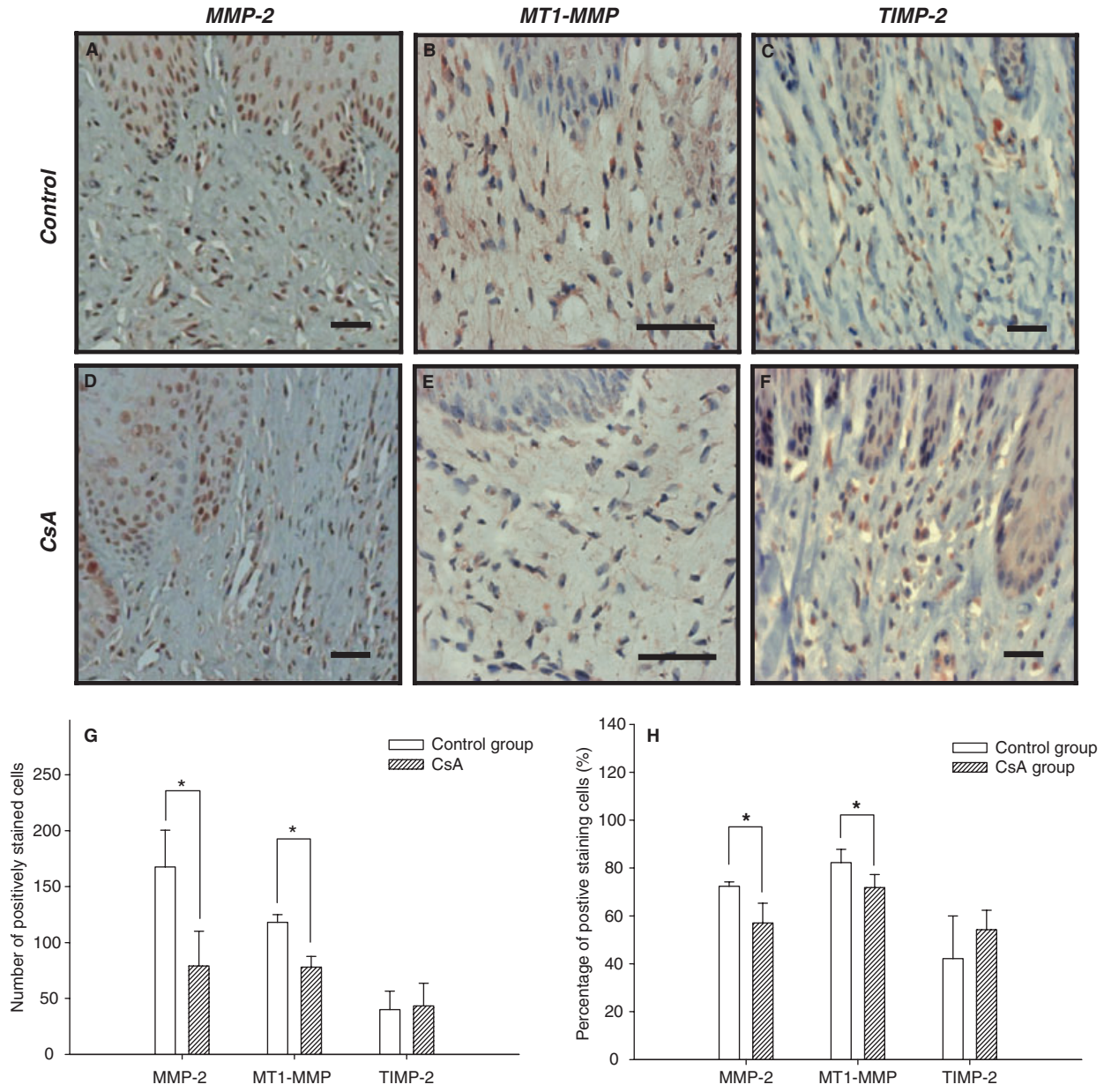

Fig. 2. Immunohistochemical staining for matrix metalloproteinase-2 (MMP-2) (A,D), membrane type-I MMP (B,E) and tissue inhibitor of metalloproteinase-2 (TIMP-2) (C,F) in gingival tissue sections from control (A–C) and cyclosporine A-treated (D–F) rats (original magnifications: ×400 for membrane type-I MMP and ×200 for TIMP-2 and MMP-2). Comparison of the numbers and percentage distributions of cells positive for MMP-2, membrane type-I MMP and TIMP-2 in the gingival stoma of control and cyclosporine A-treated rats (G,H). Data are expressed as means and standard deviations. *Significant difference between groups ($p < 0.05$; $n = 6$ for each group). CsA, cyclosporine A; MT1-MMP, membrane type-I matrix metalloproteinase.

significant effect on MMP-1 protein levels (46).

The roles of membrane type-1 MMP and TIMP-2 in cyclosporine A-induced gingival overgrowth have not been studied extensively. However, it was reported that the gelatinolytic activity of MMP-2 was reduced both in cultured fibroblasts treated with cyclosporine A and in gingival tissue of rats that received cyclosporine A (19). In contrast, increased expression of myocardial MMP-2 and vascular endothelial growth factor and myocardial fibrosis have been observed in cyclosporine A-treated rats (47). Another recent study on hereditary gingival fibromatosis revealed that TIMP-1 and TIMP-2 expression from the fibroblasts of patients with hereditary gingival fibromatosis were equivalent to those of normal gingival fibroblasts, although the hereditary gingival fibromatosis cells expressed significantly less MMP-1 and MMP-2 transcripts than normal gingival cells (48). In the present study, we observed

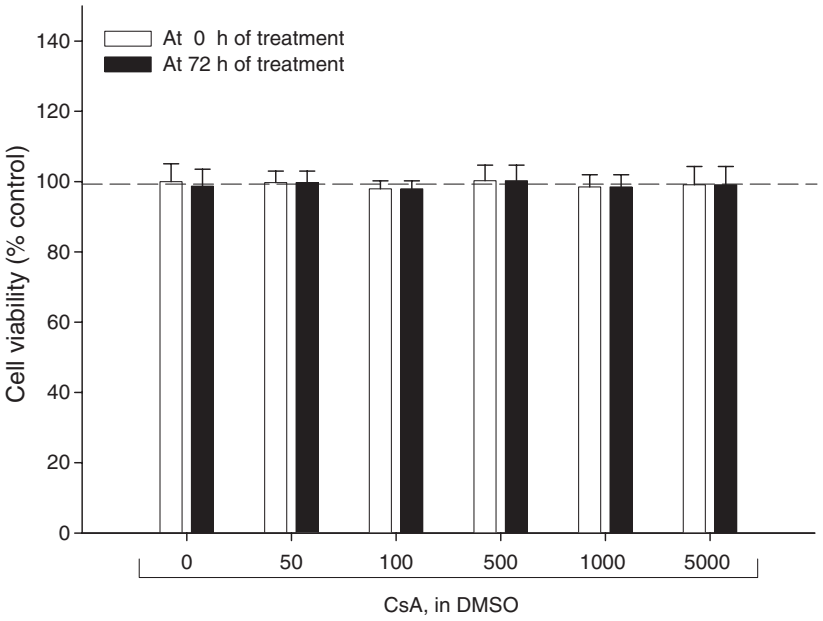

Fig. 3. Effect of cyclosporine A on the viability of human gingival fibroblasts. The proportion of viable human gingival fibroblasts from cultures with medium alone or medium containing cyclosporine A in dimethyl sulfoxide (0, 50, 100, 500, 1000 and 5000 ng/mL) was assessed using the MTS assay at 0 and 72 h after treatment. Data are expressed as means and standard deviations. CsA, cyclosporine A; DMSO, dimethylsulfoxide.

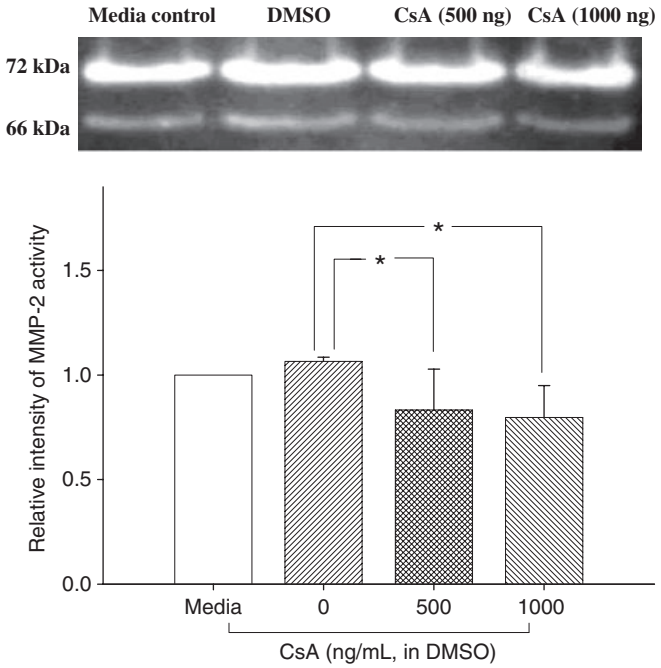

Fig. 4. Gelatinolytic activity of matrix metalloproteinase-2 (MMP-2) in gingival fibroblasts after treatment with cyclosporine A. The top part of the figure shows the pattern of gelatinolytic activity at 66 (MMP-2) and 72 (pro-MMP-2) kDa in gingival fibroblast cultures for the control and cyclosporine A-treatment groups (0, 500 and 1000 ng/mL of cyclosporine A) after 72 h. The lower part of the figure shows a comparison of the absorbance values of the active form of MMP-2 relative to that of the control. Data are expressed as means and standard deviations. *Significantly different from the control ($p < 0.05$). The experiment was repeated three times. CsA, cyclosporine A; DMSO, dimethylsulfoxide.

reduced expression of mRNA and protein for membrane type-I MMP, but not of TIMP-2, in the gingivae of cyclosporine A-treated rats (Figs 1, 2). In our *in vitro* experiment, the effect of cyclosporine A on the viability of human gingival fibroblasts was examined; our preliminarily results suggested that the viability seemed not to be affected by cyclosporine A, even at a concentration of 5000 ng/mL (Fig. 3). However, the results from zymography analyses showed a decrease of MMP-2 activity that was inversely related to the dose of CsA (Fig. 4). Although the expression of MMP-2, membrane type-I MMP and TIMP-2 mRNAs was unaffected by cyclosporine A, the ratio of membrane type-I MMP to TIMP-2 decreased dose-dependently in fibroblasts treated with cyclosporine A doses ranging from 500 to 5000 ng/mL (Fig. 5). These *in vivo* and *in vitro* results suggest that membrane type-I MMP might indirectly regulate MMP-2 activation (Fig. 6). It has been shown that at high concentrations of TIMP-2, TIMP-2 prevents MMP-2 processing by inhibiting all free membrane type-I MMP. A threshold level of TIMP-2 is necessary for construction of the ternary complex (i.e. membrane type-I MMP, TIMP-2 and pro-MMP-2), but the amount of uninhibited membrane type-I MMP present at this threshold level is still sufficient to cleave pro-MMP-2 (49–51). Our finding that the expression of TIMP-2 in human gingival fibroblasts was not affected by cyclosporine A is consistent with the results of another recent study (31); however, the ratio of TIMP-2 to membrane type-I MMP was not determined in that study.

Our study is the first to report reduced expression of membrane type-I MMP in the gingivae of rats that were treated with cyclosporine A for 4 wk. A similar finding, that membrane type-I MMP expression is suppressed by cyclosporine A, has been reported in renal glomerular mesangial cells (52). Other studies have shown that membrane type-I MMP exerts proteolytic activity against extracellular matrix components, including gelatins, by activating pro-MMP-2 on the cell surface (53–55). In the present *in vitro* study, the expres-

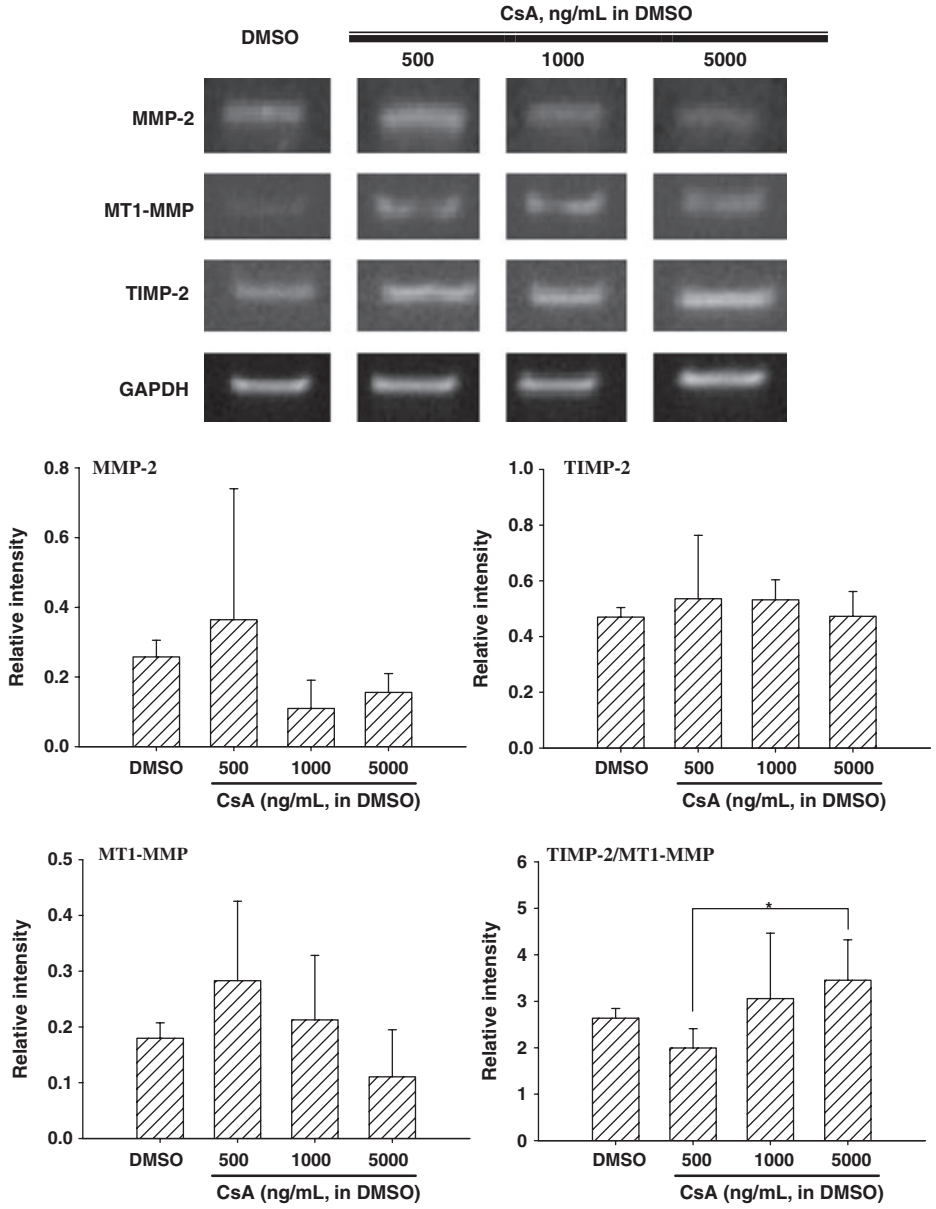

Fig. 5. The effect of cyclosporine A on the expression of matrix metalloproteinase-2 (MMP-2), membrane type-I MMP and tissue inhibitor of metalloproteinase-2 (TIMP-2) mRNA in human gingival fibroblast cultures. The top part of the figure shows the expression of mRNA in fibroblast cultures after treatment with dimethylsulfoxide and cyclosporine A (500, 1000 and 5000 ng/mL) for 72 h. The lower part of the figure shows a comparison of the relative mRNA intensities for MMP-2, membrane type-I MMP and TIMP-2 relative to that of glyceraldehyde-3-phosphate dehydrogenase. Data are expressed as means and standard deviations. *Significant difference between groups ($p < 0.05$). CsA, cyclosporine A; DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MT1-MMP, membrane type-I matrix metalloproteinase.

sion of membrane type-I MMP and TIMP-2 seemed not to be affected by cyclosporine A, but the increase in the ratio of mRNA expression of TIMP-2 to membrane type-I MMP in fibroblasts after treatment with cyclosporine A may indicate that expression of membrane type-I MMP decreased relative to that of TIMP-2. This may also indicate that the ratio of membrane type-I MMP to TIMP-2 is critical for activation of pro-MMP-2 (15–18).

Recently, extracellular matrix metalloproteinase inducer, a highly glycosylated, plasma-membrane-bound glycoprotein, has been identified and its expression is considered responsible for inducing fibroblasts to produce or secrete MMPs (56). Extracellular matrix metalloproteinase inducer, or CD147, contains a 185-amino acid extracellular domain consisting of two regions characteristic of the immunoglobin superfamily, followed by 24-amino acid residues comprising the transmembrane domain and a 39-amino acid cytoplasmic domain

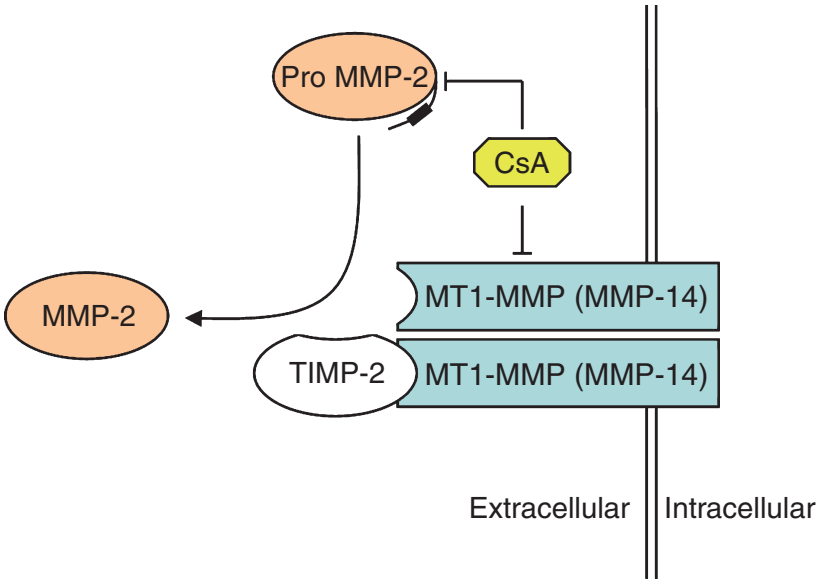

Fig. 6. The possible roles of cyclosporine A associated with the expression and activation of matrix metalloproteinase-2 (MMP-2) in gingiva. CsA, cyclosporine A; MMP-2, matrix metalloproteinase-2; MT1-MMP, membrane type-I matrix metalloproteinase; TIMP-2, tissue inhibitor of metalloproteinase-2.

(57), and was originally identified on the surfaces of tumor cells. Its expression on tumor cells may induce tumor progression and invasion by triggering the production or release of MMPs by fibroblasts and endothelial cells (58–60). Extracellular matrix metalloproteinase inducer also plays a role in cell attachment, cell migration and cell-to-cell interactions. Extracellular matrix metalloproteinase inducer stimulates production of MMP-1, MMP-2 and MMP-3, but has no effect on the TIMPs (60–62). Therefore, extracellular matrix metalloproteinase inducer may change the collagenolytic balance to favor MMP production and activation. The ability of extracellular matrix metalloproteinase inducer to stimulate MMP production suggests that this molecule may be associated with several normal and pathological tissue-modulatory processes and with tissue remodeling (61,63). Therefore, extracellular matrix metalloproteinase inducer may affect MMP-2 and membrane type-I MMP, but not TIMP-2. We recently observed decreased expression of extracellular matrix metalloproteinase inducer after cyclosporine A treatment (unpublished data, Y-T. Lu). Whether the cyclosporine A-induced decrease in gingival membrane type-I MMP and MMP-2 expression is a direct effect of the drug or an indirect response mediated by extracellular matrix metalloproteinase inducer is still unknown and requires further detailed investigations.

In conclusion, the expressions of membrane type-I MMP and TIMP-2, as well as of MMP-2, in gingivae during treatment with cyclosporine A were examined *in vivo* and *in vitro*. In the gingivae of rats, cyclosporine A significantly decreased the mRNA and protein expression of membrane type-I MMP, but not of TIMP-2. After treatment with cyclosporine A, the expression of MMP-2 mRNA was unaffected, but the expression of MMP-2 protein was decreased. In fibroblast cultures, treatment with cyclosporine A decreased MMP-2 activity in a dose-dependent manner. Although the expression of MMP-2, membrane type-I MMP and TIMP-2 mRNAs in fibroblasts was unaffected after treatment with cyclosporine A, the ratio of mRNA expression of membrane type-I MMP to that of TIMP-2 in fibroblasts decreased as the cyclosporine A dose decreased. Therefore, we suggest that cyclosporine A inhibits the expression of membrane type-I MMP in gingiva and it may further reduce the activation of MMP-2.

Acknowledgements

This study was partially supported by research grants from the National Science Council (NSC-96-2314-B-016-002) and the Department of National Defence (DOD-C97-20-02), China.

References

1. Doufexi A, Mina M, Ioannidou E. Gingival overgrowth in children: epidemiology, pathogenesis, and complications. A literature review. *J Periodontol* 2005;**76:**3–10.
2. Ono M, Hatamochi A, Arakawa M, Ueki H. Effects of cyclosporin A on cell proliferation and collagen production by human skin fibroblasts. *J Dermatol Sci* 1991;**2:**274–280.
3. Willershausen-Zonnchen B, Lemmen C, Schumacher U. Influence of cyclosporine A on growth and extracellular matrix synthesis of human fibroblasts. *J Cell Physiol* 1992;**152:**397–402.
4. Ayanoglou CM, Lesty C. Cyclosporin A-induced gingival overgrowth in the rat: a histological, ultrastructural and histomorphometric evaluation. *J Periodontal Res* 1999;**34:**7–15.
5. Mariani G, Calastrini C, Carinci F, Bergamini L, Calastrini F, Stabellini G. Ultrastructural and histochemical features of the ground substance in cyclosporin A-induced gingival overgrowth. *J Periodontol* 1996;**67:**21–27.
6. Thomason JM, Sloan P, Seymour RA. Immunolocalization of collagenase (MMP-1) and stromelysin (MMP-3) in the gingival tissues of organ transplant patients medicated with cyclosporin. *J Clin Periodontol* 1998;**25:**554–560.
7. Bode W, Gomis-Ruth FX, Stockler W. Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXX GXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. *FEBS Lett* 1993;**331:**134–140.
8. Stocker W, Grams F, Baumann U *et al.* The metzincins – topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. *Protein Sci* 1995;**4:**823–840.
9. Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. *J Pathol* 2003;**200:**448–464.

10. Nagase H, Woessner JF Jr. Matrix metalloproteinases. *J Biol Chem* 1999;**274:** 21491–21494.
11. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. *Curr Opin Cell Biol* 1998;**10:**602–608.
12. Yoshizaki T, Sato H, Furukawa M. Recent advances in the regulation of matrix metalloproteinase 2 activation: from basic research to clinical implication (Review). *Oncol Rep* 2002;**9:**607–611.
13. Sato H, Takino T, Okada Y *et al*. A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature* 1994;**370:**61–65.
14. Strongin AY, Collier I, Bannikov G, Marmer BL, Grant GA, Goldberg GI. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J Biol Chem* 1995;**270:** 5331–5338.
15. Wang Z, Juttermann R, Soloway PD. TIMP-2 is required for efficient activation of proMMP-2 in vivo. *J Biol Chem* 2000;**275:**26411–26415.
16. Hernandez-Barrantes S, Toth M, Bernardo MM *et al*. Binding of active (57 kDa) membrane type 1-matrix metalloproteinase (MT1-MMP) to tissue inhibitor of metalloproteinase (TIMP)-2 regulates MT1-MMP processing and pro-MMP-2 activation. *J Biol Chem* 2000;**275:** 12080–12089.
17. Kurschat P, Zigrino P, Nischt R *et al*. Tissue inhibitor of matrix metalloproteinase-2 regulates matrix metalloproteinase-2 activation by modulation of membrane-type 1 matrix metalloproteinase activity in high and low invasive melanoma cell lines. *J Biol Chem* 1999;**274:**21056–21062.
18. Stanton H, Gavrilovic J, Atkinson SJ *et al*. The activation of ProMMP-2 (gelatinase A) by HT1080 fibrosarcoma cells is promoted by culture on a fibronectin substrate and is concomitant with an increase in processing of MT1-MMP (MMP-14) to a 45 kDa form. *J Cell Sci* 1998;**18:**2789–2798.
19. Bolzani G, Della Coletta R, Martelli Junior H, Graner E. Cyclosporin A inhibits production and activity of matrix metalloproteinases by gingival fibroblasts. *J Periodontal Res* 2000;**35:**51–58.
20. Brook NR, Waller JR, Bicknell GR, Nicholson ML. The novel antifibrotic agent pirfenidone attenuates the profibrotic environment generated by calcineurin inhibitors in the rat salt-depletion model. *Transplant Proc* 2005; **37:**130–133.
21. Silva HC, Coletta RD, Jorge J, Bolzani G, de Almeida OP, Graner E. The effect of cyclosporin A on the activity of matrix metalloproteinases during the healing of rat molar extraction wounds. *Arch Oral Biol* 2001;**46:**875–879.
22. Young BA, Burdmann EA, Johnson RJ *et al*. Cellular proliferation and macrophage influx precede interstitial fibrosis in cyclosporine nephrotoxicity. *Kidney Int* 1995;**48:**439–448.
23. Bonnaure-Mallet M, Tricot-Doleux S, Godeau GJ. Changes in extracellular matrix macromolecules in human gingiva after treatment with drugs inducing gingival overgrowth. *Arch Oral Biol* 1995;**40:**393–400.
24. Pattamapun K, Tiranathanagul S, Yongchaitrakul T, Kuwatanasuchat J, Pavasant P. Activation of MMP-2 by *Porphyromonas gingivalis* in human periodontal ligament cells. *J Periodontal Res* 2003;**38:**115–121.
25. Tiranathanagul S, Pattamapun K, Yongchaitrakul T, Pavasant P. MMP-2 activation by *Actinobacillus actinomycetemcomitans* supernatant in human PDL cells was corresponded with reduction of TIMP-2. *Oral Dis* 2004;**10:**383–388.
26. Gronert K, Kantarci A, Levy BD *et al*. A molecular defect in intracellular lipid signaling in human neutrophils in localized aggressive periodontal tissue damage. *J Immunol* 2004;**172:**1856–1861.
27. Chen YT, Tu HP, Chin YT *et al*. Upregulation of transforming growth factor-beta1 and vascular endothelial growth factor gene and protein expression in cyclosporin-induced overgrown edentulous gingiva in rats. *J Periodontol* 2005; **76:**2267–2275.
28. 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.
29. Slattery C, Campbell E, McMorrow T, Ryan MP. Cyclosporine A-induced renal fibrosis: a role for epithelial-mesenchymal transition. *Am J Pathol* 2005;**167:** 395–407.
30. Overall CM, Tam E, McQuibban GA *et al*. Domain interactions in the gelatinase A.TIMP-2.MT1-MMP activation complex. The ectodomain of the 44-kDa form of membrane type-1 matrix metalloproteinase does not modulate gelatinase A activation. *J Biol Chem* 2000;**275:** 39497–39506.
31. Cotrim P, de Andrade CR, Martelli-Junior H, Graner E, Sauk JJ, Coletta RD. Expression of matrix metalloproteinases in cyclosporin-treated gingival fibroblasts is regulated by transforming growth factor (TGF)-beta1 autocrine stimulation. *J Periodontol* 2002;**73:**1313–1322.
32. 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.
33. Fu E, Nieh S, Chang HL, Wang SL. Cyclosporin A-induced gingival overgrowth in rats: macroscopic and microscopic observations. *Int J Periodontics Restorative Dent* 1996;**16:**278–291.
34. Iacopino AM, Doxey D, Cutler CW *et al*. Phenytoin and cyclosporine A specifically regulate macrophage phenotype and expression of platelet-derived growth factor and interleukin-1 in vitro and in vivo: possible molecular mechanism of drug-induced gingival hyperplasia. *J Periodontol* 1997;**68:**73–83.
35. Nares S, Ng MC, Dill RE, Park B, Cutler CW, Iacopino AM. Cyclosporine A upregulates platelet-derived growth factor B chain in hyperplastic human gingiva. *J Periodontol* 1996;**67:**271–278.
36. Morton RS, Dongari-Bagtzoglou AI. Regulation of gingival fibroblast interleukin-6 secretion by cyclosporine A. *J Periodontol* 1999;**70:**1464–1471.
37. Myrillas TT, Linden GJ, Marley JJ, Irwin CR. Cyclosporin A regulates interleukin-1beta and interleukin-6 expression in gingiva: implications for gingival overgrowth. *J Periodontol* 1999;**70:**294–300.
38. Williamson MS, Miller EK, Plemons J, Rees T, Iacopino AM. Cyclosporine A upregulates interleukin-6 gene expression in human gingiva: possible mechanism for gingival overgrowth. *J Periodontol* 1994; **65:**895–903.
39. Tipton DA, Stricklin GP, Dabbous MK. Fibroblast heterogeneity in collagenolytic response to cyclosporine. *J Cell Biochem* 1991;**46:**152–165.
40. Tipton DA, Pabst MJ, Dabbous MK. Interleukin-1 beta- and tumor necrosis factor-alpha-independent monocyte stimulation of fibroblast collagenase activity. *J Cell Biochem* 1990;**44:**253–264.
41. Gagliano N, Moscheni C, Dellavia C *et al*. Effect of cyclosporin A on human gingival fibroblast collagen turnover in relation to the development of gingival overgrowth: an in vitro study. *Biomed Pharmacother* 2004;**58:**231–238.
42. Reynolds JJ, Meikle MC. The functional balance of metalloproteinases and inhibitors in tissue degradation: relevance to oral pathologies. *J R Coll Surg Edinb* 1997;**42:**154–160.
43. Yamada H, Nishimura F, Naruishi K *et al*. Phenytoin and cyclosporin A suppress the expression of MMP-1, TIMP-1, and cathepsin L, but not cathepsin B in cultured gingival fibroblasts. *J Periodontol* 2000;**71:**955–960.
44. Hyland PL, Traynor PS, Myrillas TT *et al*. The effects of cyclosporin on the collagenolytic activity of gingival fibroblasts. *J Periodontol* 2003;**74:**437–445.
45. Yamaguchi M, Naruishi K, Yamada-Naruishi H, Omori K, Nishimura F, Takashiba S. Long-term cyclosporin A

exposure suppresses cathepsin-B and -L activity in gingival fibroblasts. *J Periodontal Res* 2004;**39:**320–326.

46. Tuter G, Serdar MA, Yalim M, Gurhan IS, Balos K. Evaluation of matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 levels in gingival fibroblasts of cyclosporin A-treated patients. *J Periodontol* 2002;**73:**1273–1278.
47. Bianchi R, Rodella L, Rezzani R. Cyclosporine A up-regulates expression of matrix metalloproteinase 2 and vascular endothelial growth factor in rat heart. *Int Immunopharmacol* 2003;**3:**427–433.
48. Coletta RD, Almeida OP, Reynolds MA, Sauk JJ. Alteration in expression of MMP-1 and MMP-2 but not TIMP-1 and TIMP-2 in hereditary gingival fibromatosis is mediated by TGF-beta 1 autocrine stimulation. *J Periodontal Res* 1999; **34:**457–463.
49. Butler GS, Hutton M, Wattam BA *et al*. The specificity of TIMP-2 for matrix metalloproteinases can be modified by single amino acid mutations. *J Biol Chem* 1999;**274:**20391–20396.
50. Jo Y, Yeon J, Kim HJ, Lee ST. Analysis of tissue inhibitor of metalloproteinases-2 effect on pro-matrix metalloproteinase-2 activation by membrane-type 1 matrix metalloproteinase using baculovirus/insect-cell expression system. *Biochem J* 2000;**3:**511–519.
51. Sato H, Kinoshita T, Takino T, Nakayama K, Seiki M. Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2. *FEBS Lett* 1996;**393:**101–104.
52. Alfonso-Jaume MA, Mahimkar R, Lovett DH. Co-operative interactions between NFAT (nuclear factor of activated T cells) c1 and the zinc finger transcription factors Sp1/Sp3 and Egr-1 regulate MT1-MMP (membrane type 1 matrix metalloproteinase) transcription by glomerular mesangial cells. *Biochem J* 2004;**380:**735–747.
53. Tsunezuka Y, Kinoh H, Takino T *et al*. Expression of membrane-type matrix metalloproteinase 1 (MT1-MMP) in tumor cells enhances pulmonary metastasis in an experimental metastasis assay. *Cancer Res* 1996;**56:**5678–5683.
54. Imai K, Ohuchi E, Aoki T *et al*. Membrane-type matrix metalloproteinase 1 is a gelatinolytic enzyme and is secreted in a complex with tissue inhibitor of metalloproteinases 2. *Cancer Res* 1996;**56:**2707–2710.
55. Pei D, Weiss SJ. Transmembrane-deletion mutants of the membrane-type matrix metalloproteinase-1 process progelatinase A and express intrinsic matrix-degrading activity. *J Biol Chem* 1996;**271:**9135–9140.
56. Foda HD, Rollo EE, Drews M *et al*. Ventilator-induced lung injury upregulates and activates gelatinases and EMMPRIN: attenuation by the synthetic matrix metalloproteinase inhibitor, Prinomastat (AG3340). *Am J Respir Cell Mol Biol* 2001;**25:**717–724.
57. Biswas C, Zhang Y, DeCastro R *et al*. The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. *Cancer Res* 1995;**55:**434–439.
58. Bordador LC, Li X, Toole B *et al*. Expression of emmprin by oral squamous cell carcinoma. *Int J Cancer* 2000;**85:**347–352.
59. Kanekura T, Chen X, Kanzaki T. Basigin (CD147) is expressed on melanoma cells and induces tumor cell invasion by stimulating production of matrix metalloproteinases by fibroblasts. *Int J Cancer* 2002;**99:**520–528.
60. Zucker S, Hymowitz M, Rollo EE *et al*. Tumorigenic potential of extracellular matrix metalloproteinase inducer. *Am J Pathol* 2001;**158:**1921–1928.
61. Guo H, Zucker S, Gordon MK, Toole BP, Biswas C. Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. *J Biol Chem* 1997;**272:**24–27.
62. Sun J, Hemler ME. Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interactions. *Cancer Res* 2001;**61:** 2276–2281.
63. Gabison EE, Hoang-Xuan T, Mauviel A, Menashi S. EMMPRIN/CD147, an MMP modulator in cancer, development and tissue repair. *Biochimie* 2005;**87:**361–368.