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Cyclosporine A enhances apoptosis in gingival keratinocytes of rats and in OECM1 cells via the mitochondrial pathway

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Background and Objective: We reported previously that cyclosporine A induces a high level of expression of p21 in rat gingival keratinocytes and in OECM1 cells. In this study, the apoptosis of gingival keratinocytes after treatment with cyclosporine A was evaluated using the same models.

Material and Methods: Forty Sprague–Dawley rats with right edentulous ridges were assigned into cyclosporine A (30 mg/kg) and control groups. Four weeks later, gingivae were screened for expression of apoptotic genes using microarray analyses and DNA fragmentation. The expression of bcl2-associated X protein (Bax), apoptosis-inducing factor (AIF) and Caspase 3 mRNAs, and the expression of Bax, AIF, Caspase 9 and Fas proteins, were analyzed using the reverse transcription–polymerase chain reaction and immunohistochemistry, respectively. Apoptosis in OECM1 cells (keratinocytes of a gingival carcinoma cell line), after treatment with cyclosporine A, was evaluated by 4′,6-diamidino-2-phenylindole (DAPI) staining and flow cytometry, whereas the expression of Bax, AIF, Caspase 3 and 8, Bcl-2 and Fas proteins were examined using western blotting.

Results: According to microarray analyses, the expression of certain apoptotic genes was altered in the gingiva of rats who received cyclosporine A, and increased number of DNA fragments were detected. Expression of mRNA or protein for Bax, AIF and Caspase 3 and 9 in the gingivae of rats increased after treatment with cyclosporine A. An increased number of apoptotic bodies and of OECM1 cells in the sub-G1 phase was observed after treatment with cyclosporine A. Increased expression of AIF, Bax and Caspase 3 protein, but not of bcl-2, Caspase 8 or Fas protein, was observed in cells after treatment with cyclosporine A.

Conclusion: Based on the above findings, we suggest that cyclosporine A might enhance the apoptosis of gingival keratinocytes, mainly via the mitochondrial pathway.

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Key words: cyclosporine A; gingiva; keratinocyte; apoptosis

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Systemic administration of cyclosporine A can induce gingival overgrowth (1,2). This effect may be related to the hyperplasia of gingival keratinocytes induced by cyclosporine A (3,4). Studies have found that cyclosporine A up-regulates epithelial growth factor and keratinocyte growth factor (5,6), both of which are produced by

epithelial cells. In our recent study, higher expressions of p21 mRNA and protein, via a p53-independent pathway, were observed after treatment with cyclosporine A (7). Protein p21 is the founding member of the family of cyclin-dependent kinase inhibitors, which also includes p27 and p57 (8). Protein p21 plays an essential role in growth arrest (9), and its over-expression leads to G1 and G2 (10) or S-phase arrest (11). In addition to regulating normal cell cycle progression, p21 integrates genotoxic signal insults into apoptotic signaling pathways that ultimately determine cell fate (12). Therefore, a detailed investigation of gingival keratinocyte stasis or apoptosis during cyclosporine A therapy is of interest.

A previous study has shown that apoptosis, the programmed cell death, is involved with the shedding of normal gingival keratinocytes (13). Two main pathways for apoptosis have been defined: the extrinsic pathway, which results from activation of death receptors; and an intrinsic pathway that may result from mitochondrial or endoplasmic reticulum stress. Engagement of death receptors, such as Fas, leads to activation of Caspase 8 and subsequent apoptosis (14). In this regard, cyclosporine A increases Fas expression in cultured tubular cells, and increased FasL and Fas expression has been reported in chronic cyclosporine A nephrotoxicity (15,16). Both ligand-dependent and ligand-independent Fas activation have been implicated in drug cytotoxicity (17,18). Mitochondrial injury leads to the release of apoptosis mediators, such as cytochrome *c* and Smac/Diablo, and to the loss of mitochondrial transmembrane potential (19). Release of cytochrome *c* facilitates Caspase 9 activation, subsequent activation of effector caspases, such as Caspase 3, and apoptosis. More recently, endoplasmic reticulum stress has been defined as an activator of apoptosis (20).

Studies have suggested that epidermal keratinocytes, hair epithelial cells, renal tubule epithelial cells and lung epithelial cells are sensitive to the action of cyclosporine A (15,21–23). The response of particular tissues to cyclosporine A, however, seems dependent on both the cell type and their structural relationships within the tissue (23). Cyclosporine A inhibited the proliferation of cells in renal tubule epithelial cell lines through apoptosis was reported (15,24). In gingiva, the effect of cyclosporine A on cell apoptosis has been investigated. Decreased levels of Caspase 3 in gingival keratinocytes (25), and a lower apoptosis grade in patients with cyclosporine A-induced gingival overgrowth than those in the control of gingivitis (26) were observed. A similar extent of keratinocyte apoptosis in the gingiva of kidney transplantation recipients with cyclosporine A-induced gingival overgrowth was reported in another study (27). It was further found that cyclosporine A inhibited oral epithelial cell division, but this effect was not associated with changes in apoptosis in the primarily cultured oral keratinocytes and cell lines (28). Thus, the exact role of cyclosporine A on the apoptosis of gingival keratinocytes is still uncertain, although hyperplastic keratinocytes are consistently observed in overgrown gingiva. In the present study, the effect of cyclosporine A on the apoptosis of keratinocytes in the gingiva, via the death receptor or mitochondrial pathways, was examined by using the *in vivo* and *in vitro* models as in our previous studies (6,7).

Material and methods

In vivo experiment

Forty male 5-wk-old Sprague–Dawley rats, weighing 120–150 g, were used in the study. The rats were randomly assigned to cyclosporine A and control groups after a 3-wk healing period following the extraction of all maxillary right molars, as in our previous study (4). Animals in the cyclosporine A group received cyclosporine A (Sandimmun; Sandoz, Basel, Switzerland) (30 mg/kg body weight in mineral oil) daily by gastric feeding for 4 wk, whereas the control group rats received mineral oil alone. At the end of the study, all animals were killed by carbon dioxide inhalation. The edentulous gingival specimens from five rats in each group were collected and pooled for microarray analyses, in which an initial screen of the mRNA expressions for cell apoptosis was performed. Ten edentulous specimens in each group were immediately frozen in liquid nitrogen and stored at −70°C until use. As a complementary approach, internucleosomal DNA fragmentation in each gingival spaceman (six in each group) was quantitatively assayed by antibody-mediated capture and detection of cytoplasmic mononucleosome-associated and oligonucleosome-associated histone–DNA complexes (Cell Death Detection ELISA^{plus} kit; Roche Diagnostics GmbH, Mannheim, Germany) that accumulated in dying cells with an intact cell membrane (29). Then, the mRNA expressions of bcl2-associated X protein (Bax), apoptosis-inducing factor (AIF) and Caspase 3 in the remaining stored specimens (four in each group) were examined using the reverse transcription–polymerase chain reaction (RT-PCR). In this *in vivo* experiment, five fresh gingival specimens from each group were also obtained. After fixation in 4% paraformaldehyde and paraffin embedding, the serial tissue sections were sliced buccopalatally to a thickness of 4 μm and analyzed using immunohistochemistry to evaluate the expression of Bax, AIF, Fas and Caspase 9 protein.

Microarray assay — After homogenizing, the total RNA of edentulous tissue was extracted using Trizol reagent and quantified by spectrophotometry at 260 nm. The tissue RNA was reverse transcribed into cDNA, *in vitro* transcribed into cRNA and then labeled with CyDye using the MessageAmp aRNA Kit (Ambion, Houston, TX, USA), according to the manufacturer's protocol. cRNA obtained from the RNA sample of the control group was labeled with Cy3 and acted as the reference sample. cRNA obtained from the RNA sample of the cyclosporine A group was labeled with Cy5 and acted as the experimental sample. Labeled aRNA of reference and experimental samples was purified to remove uncoupled CyDyes, combined in equal amounts

and mixed with 2× hybridization buffer, according to the manufacturer's protocol, before being applied onto the microarray. Hybridization was performed on the Rat Oligo 1A Microarray (G4130A) (Agilent Technologies, Santa Clara, CA, USA) containing about 22,000 oligonucleotides representing more than 17,000 genes. Conditions of hybridization and washing were followed according to the Agilent 60-mer oligo microarray processing protocol (Agilent Technologies).

RNA extraction and RT-PCR — Total RNAs of homogenized gingival tissue were extracted using Trizol reagent and quantified by spectrophotometry at 260 nm. Using the PCR system (GeneAmp®-9700; Applied Biosystems, Foster City, CA, USA) (at 55°C for 1 h), 5 μg of total RNAs were reverse transcribed, using Superscript III, into total cDNA, which was used as a template for PCR reactions and analysis. The PCR reactions involved an initial denaturation at 94°C for 2 min 30 s, followed by 30 or 35 cycles at 94°C for 30 s, exposure to an appropriate annealing temperature (58–62°C) for 30 s, and then a final incubation at 72°C for 60 s. The PCR primers for analysis of mRNA in gingival tissue and fibroblast samples were: Bax, sense (5′-GAATATGACGCACGGATCGTT-3′) and antisense (5′-TGCTCCCAGATGTTTGGAGCT-3′); AIF, sense (5′-GTACTGAAGAGCAGCTAAGGCG-3′) and antisense (5′-TTCTAACGTGTCAAACCCTGGA-3′); Caspase 3, sense (5′-AGAAGATGGTTTGAGCCGGA-3′) and antisense (5′-TTAAGGAAGCCTGGAGCACAG-3′); rat glyceraldehyde-3-phosphate dehydrogenase, sense (5′-TGCTGGTGCTGAGTATGTCG-3′) and antisense (5′-ATTGAGAGCAATGCCAGCC-3′). All PCR primers described above were designed and supplied by Seeing Bioscience Co. Ltd (Taipei, Taiwan). The exponential phases of the RT-PCR amplifications were determined over 30–35 cycles to allow quantitative comparisons between the cDNAs. Amplified RT-PCR products were then analyzed on 1% agarose gels and visualized using ethidium bromide staining and a camera system (Transilluminator/SPOT; Diagnostic Instruments, Sterling Heights, MI, USA). The gel images of the RT–PCR products were directly scanned (ONE-Dscan 1-D Gel Analysis Software; Scanalytic Inc. Fairfax, VA, USA), and the relative densities were obtained by determining the ratio of the signal intensity to the glyceraldehyde-3-phosphate dehydrogenase band. Gene expression between the test (cyclosporine A treated) and the control groups was compared.

DNA fragmentation — As a complementary approach, internucleosomal DNA fragmentation was quantitatively assayed using antibody-mediated capture and detection of cytoplasmic mononucleosome-associated and oligonucleosome-associated histone–DNA complexes (Cell Death Detection ELISA plus kit; Roche Molecular Biochemicals, Mannheim, Germany) that accumulated in dying neutrophils with an intact cell membrane (29). Briefly, gingival specimens were washed, resuspended in 200 μL of the lysis buffer supplied by the manufacturer and then incubated for 30 min at room temperature (25°C). After pelleting nuclei (200 *g*, 10 min), 20 μL of the supernatant (cytoplasmic fraction) was used in the enzyme-linked immunosorbent assay (ELISA) following the manufacturer's standard protocol. Finally, the absorbance at 405 and 490 nm (reference wavelength), upon incubation with a peroxidase substrate for 5 min, was determined using a microplate reader (Bio-Tec Instruments, Winooski, VT, USA). Signals in the wells containing the substrate only were subtracted as background.

Immunohistochemistry — After deparaffinization and hydration, tissue sections were boiled in DAKO buffer (DAKO target retrieval solution; DAKO North American Inc., Real Carpinteria, CA, USA) for 15 min for antigen retrieval. Endogenous peroxidase activity was quenched by incubation for 5 min with 0.1% hydrogen peroxide in distilled water. Tissue sections were washed twice, for 5 min each wash, in phosphate-buffered saline. Then, the sections were incubated for 2 h with unconjugated primary polyclonal antibodies against Bax, AIF, Caspase 9, and Fas (Santa Cruz; Santa Cruz, CA, USA), followed by further incubation with biotinylated secondary antibody, streptavidin-conjugated horseradish peroxidase complexes and 3-amino-9-ethyl carbazole solution (Dako Cytomation, San Diego, CA, USA) for a further 30, 30 and 10 min, respectively. Between incubations, the cells and sections were washed with sterile phosphate-buffered saline. The specimens were then counterstained with hematoxylin, dehydrated and mounted. The cells that stained positively for Bax, AIF, Caspase 9 and Fas were examined using microscopy.

In vitro study

Keratinocytes of a human gingival squamous carcinoma cell line (OECM1), a generous gift from Dr Ching-Liang Meng of the National Defense Medical Center, was grown in RPMI-1640 (GibcoBRL; Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 50 U/mL of penicillin G, 50 mg/mL of streptomycin sulfate and 1.25 mg/mL of amphotericin B (GibcoBRL, Life Technologies) (6). The cells were cultured in a humidified atmosphere, of 95% air and 5% CO_2, at 37°C overnight until the cells were about 70% confluent. Before treatment with cyclosporine A, the cells were rinsed with sterile phosphate-buffered saline. The cells were then maintained overnight in culture medium containing 2% fetal bovine serum and were then stimulated with different concentrations (0, 2, 10, 100 and 1000 ng/mL) of cyclosporine A (Sandimmun) in dimethylsulfoxide (Sigma®; Sigma-Aldrich. Inc., St Louis, MO, USA) for up to 32 h. The apoptotic bodies formed in the OECM1 cells, cultured in the presence or absence of cyclosporine A, were stained with 4′,6-diamidino-2-phenylindole (DAPI) and examined under a fluorescent microscope. Cell cycle analysis, particularly regarding the sub-G1 phase, of OECM1 cells after cyclosporine A

therapy (10^3 ng/mL for 32 h), was examined using flow cytometry. The expression of proteins associated with the cell death receptor pathway (Fas and Caspase 8), the mitochondrial pathway (Bax, Bcl-2 and AIF), as well as the common pathway (Caspase 3), were examined using western blotting.

Flow cytometry — The OECM1 cells, after stimulation with cyclosporine A for 32 h, were treated with EDTA–trypsin. The cell suspension was diluted with phosphate-buffered saline and centrifuged (about 100 *g*, 5 min, 4°C) in a Beckman J-6B centrifuge (Beckman Coulter Inc., Fullerton, CA, USA). The cell pellet was resuspended in 1.0 mL of phosphate-buffered saline and fixed by the addition of 4 mL of absolute ethanol cooloed to −20°C. These cells were stored at −20°C until required for analysis. To prepare the fixed cells for flow cytometry, they were centrifuged and resuspended in 1.0 mL of phosphate-buffered saline. A total of 100 μL of 200 μg/mL RNase A (Sigma Chemical Co., St Louis, MO, USA), which had been treated by boiling for 5 min to remove DNAse, was added and the suspension was incubated at 37°C for 30 min. One-hundred microliters of 1 mg/mL propidium iodide (Sigma Chemical Co.) was added and the suspension was incubated at room temperature for 5–10 min. The stained cell suspension was diluted 10-fold in phosphate- buffered saline immediately before analysis by flow cytometry (Ortho Diagnostic System Model 50H; Ortho Diagnostic Systems, Westwood, MA, USA). Cell cycle analysis was carried out using the multicycle data-analysis package (Phoenix Flow Systems, San Diego, CA, USA) (30).

Western blotting — Homogenates of OECM1 cells, lysed in lysis buffer, were centrifuged (13,000 *g*, 4°C, 15 min) and boiled at 100°C for 10 min. Protein concentrations were determined using a protein microassay of the BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) and separated by sodium dodecyl sulfate–polyacrylamide gele electrophoresis in 15% polyacrylamide gels and then electoblotted/transferred onto poly (vinylidene difluoride) membrane. Nonspecific proteins were blocked by incubating the blots for 1 h in 5% fetal calf serum. After six washes in phosphate-buffered saline containing 0.005% Tween 20, Fas, Caspase 8, Bax, Bcl-2, AIF and Caspase 3 (Santa Cruz) proteins were detected by incubation with primary antibody (31), overnight at 4°C, followed by incubation with secondary antibody (goat anti-mouse or anti-rabbit) IgG–horseradish peroxidase conjugate at a dilution of 1 : 5000 for 1 h. Antibody-reactive proteins were detected using enhanced chemiluminescence. The expressions of AIF, Caspase 3, Bax, Bcl-2, Caspase 8 and Fas in cyclosporine A-treated OECM1 cells were presented as proportional increases or deceases relative to the levels observed in the control cell.

Statistical analysis

The Student's *t*-test was used to evaluate the differences between the control group and the cyclosporine A group regarding the expression of mRNA for Bax, AIF, Caspase 3 and glyceraldehyde-3-phosphate dehydrogenase, as determined using RT-PCR and in the DNA fragments of gingival tissue. A *p*-value of < 0.05 was selected as the significant level.

Results

In gingivae of cyclosporine A-treated rats, the Cy5/Cy3 ratio of mRNA fluorescence increased in certain genes associated with cell apoptosis [including Bax (fluorescence ratio = 1.24), AIF (fluorescence ratio = 1.27), Caspase 3 (fluorescence ratio = 1.18) and rat programmed cell death 10 (fluorescence ratio = 1.25)], compared with the Cy5/Cy3 ratio of mRNA fluorescent intensities in control rats, as determined using microarray (Table 1); however, the ratio for apoptosis inhibitor protein 3 (fluorescence ratio = 0.76) was deceased. The transcription of genes for rat transforming growth factor beta-1 and vascular endothelial growth factor was also increased (1.25-fold and 1.27-fold increase for transforming growth factor beta-1 and vascular endothelial growth factor, respectively). Increased number of DNA fragments were found in edentulous gingivae of cyclosporine A-treated rats compared with the

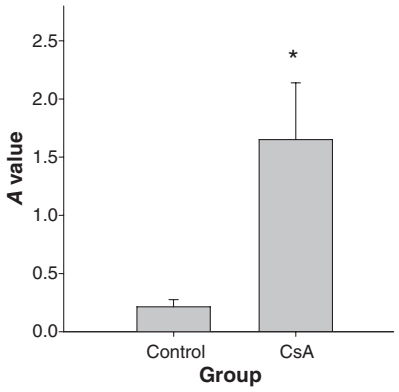


Fig. 1. DNA fragments in gingival edentulous tissue of control rats and of cyclosporine A-treated rats. (Data are presented as means and standard deviations. *Significant difference from control rats at a *p*-value of < 0.05). *A*, absorbance; CsA, cyclosporine A.

Table 1. The ratio of mRNA fluorescent intensities for Cy5/Cy3 in genes associated with cell apoptosis, as determined using microarray analysis

Gene name (Unigene)	GenBank accession no.	Fold change
Rat bcl2-associated X protein (Bax)	NM_017059	1.24
Rat programmed cell death 8 (apoptosis- inducing factor, AIF)	NM_031356	1.27
Rat Caspase 3, apoptosis related cysteine protease (Casp3)	NM_012922	1.18
Rat programmed cell death 10	CB545171	1.25
Rat apoptosis inhibitor protein 3 (Api3)	NM_022231	0.76
Rat transforming growth factor beta-1	NM_021578	1.25
Rat vascular endothelial growth factor	NM_031836	1.27

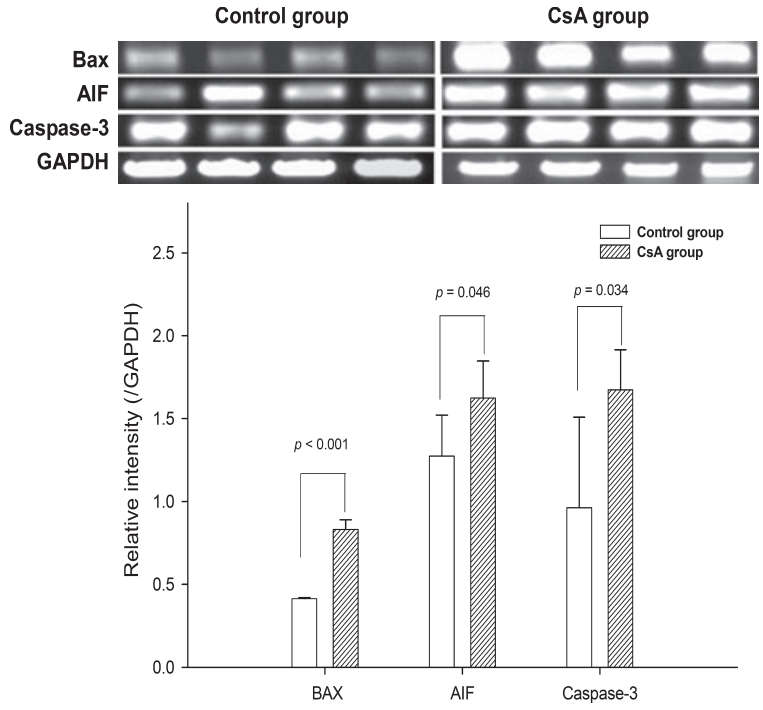


Fig. 2. The expression of bcl2-associated X protein (Bax), apoptosis-inducing factor (AIF) and Caspase 3 mRNAs in gingival edentulous tissue of control and of cyclosporine A-treated rats. The top part of the figure shows the expression of mRNA for Bax, AIF and Caspase 3 in four gingival tissues from control and cyclosporine A groups. The bottom part of the figure shows a comparison of the relative densities of mRNAs for Bax, AIF and Caspase 3 with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) between gingival tissues from control and cyclosporine A groups. (Data are presented as means and standard deviations. Significant difference from control rats was selected when the p-value < 0.05). CsA, cyclosporine A.

gingivae of control rats (Fig. 1). The results of RT-PCR analyses showed that the expression of Bax, AIF and Caspase 3 mRNAs increased in gingivae from the cyclosporine A-treated rats compared with that from the control rats (Fig. 2). The results of immunohistochemistry analysis showed stronger expression of Bax, AIF and Caspase 9 proteins in gingivae from cyclosporine A-treated rats than in gingivae from control rats, but a similar expression of Fas in the gingivae from animals of the two experimental groups (Fig. 3).

In OECM1 cells, the apoptotic bodies were easily observed by DAPI staining after treatment with cyclosporine A (Fig. 4). Flow cytometry analysis showed that the cell cycle distributions of OECM1 cells were changed when cells were treated with cyclosporine A (Fig. 5). The proportion of cells in the sub-G1 (M1) phase, representing the apoptotic cells, clearly increased after treatment with cyclosporine A when compared with no cyclosporine A treatment. Enhanced expressions of AIF protein (at cyclosporine A concentrations greater than 2 ng/mL), Caspase 3 protein (at cyclosporine A concentrations greater than 2 ng/mL) and Bax protein (at 2, 10 and 100 ng/mL of cyclosporine A) were observed by western blotting of OECM1 cells treated with cyclosporine A, while the expressions of Fas, Caspase 8 and Bcl-2 were unchanged (Fig. 6).

Discussion

In this study, the expression of genes and proteins associated with cell apoptosis were examined in the edentulous gingiva of rats and in OECM1 cells, a gingival squamous carcinoma cell line of humans, following treatment with cyclosporine A. Cell apoptosis was initially screened using microarray and further evaluated by DNA fragmentation in gingiva of rat (an *in vivo* experiment). Our results showed that the ratios of certain genes related to apoptosis were altered and that the number of DNA fragments was increased following treatment with cyclosporine A. In OECM1 cells (an *in vitro* experiment), apoptotic bodies were easily observed following DAPI staining after treatment with 1000 ng/mL of cyclosporine A (Fig. 4) and apoptosis was further confirmed by flow cytometry analysis, which showed an increased number of cells in the sub-G1 phase (Fig. 5). Based on the above findings, the signals of apoptosis related to mitochondrial or death receptor pathways were examined. The expression of signals in the mitochondrial pathway (Bax and AIF) and in the common pathway (Caspase 3) were increased after cyclosporine A treatment, whereas the expression of signals in the death receptor pathway (Fas and Caspase 8) was similar between the cyclosporine A-treated and control groups in our *in vivo* model. In the *in vitro* experiment (using OECM1 cells), similar findings of enhanced expression of AIF, Caspase 3 and Bax proteins after treatment with cyclosporine A were once again confirmed by western blotting. Based on these findings, we suggest that Bax-mediated mitochondrial injury might be the main pathway for apoptosis of gingival keratinocytes during treatment with cyclosporine A.

Similarly to our findings, the apoptosis of renal tubular cells, involving the mitochondrial pathway, was proposed to contribute to cyclosporine A-induced nephrotoxicity (24). In that study, the cultured murine renal tubular epithelial cells constitutively expressed FasL, whereas treatment with cyclosporine A increased the expression of Fas. However, Fas had no role in cyclosporine A-induced apoptosis, as cyclosporine A did not sensitize to FasL-induced apoptosis, Caspase 8 activity was not increased, and neither blocking anti-FasL serum nor Caspase 8 inhibition prevented cyclosporine A-induced apoptosis. Apoptosis induced by cyclosporine A is associated with the translocation of Bax to the mitochondria, and Bax antisense oligodeoxynucleotides protected from

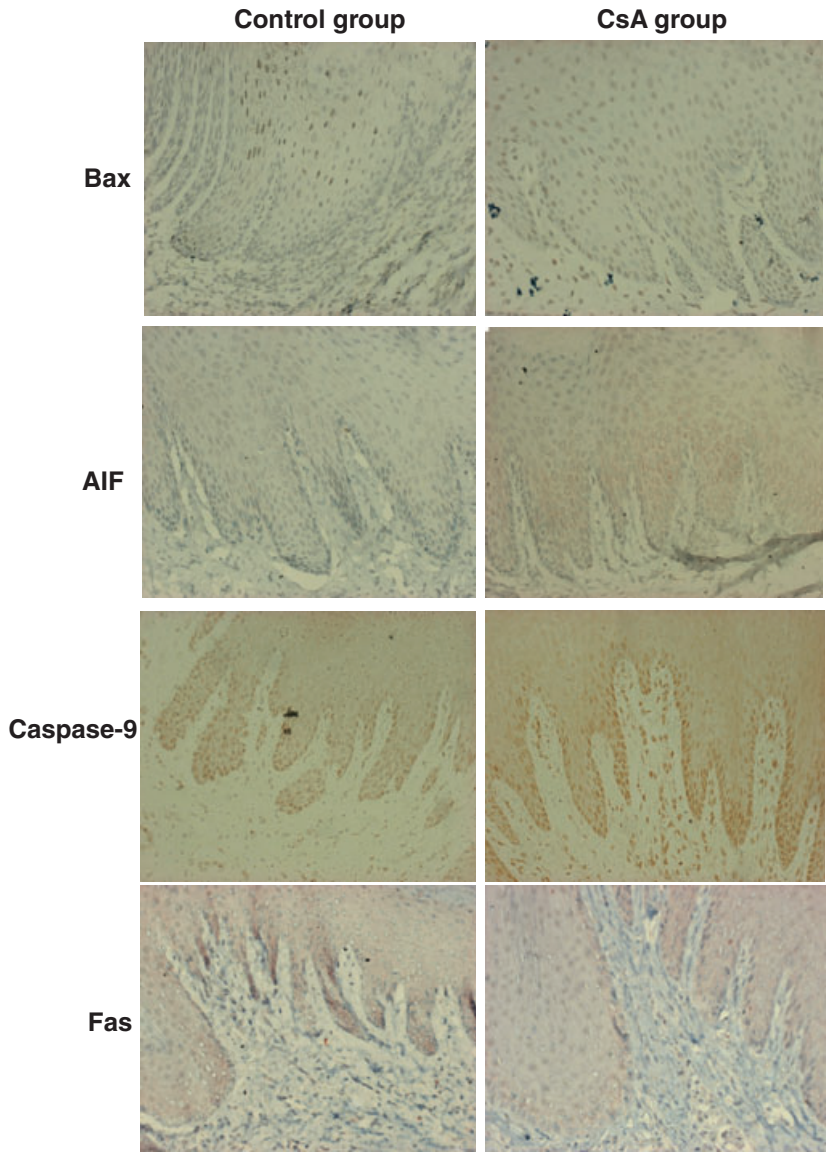


Fig. 3. Microphotographs showing immunohistochemistry staining for bcl2-associated X protein (Bax), apoptosis-inducing factor (AIF), Caspase 9 and Fas in gingival tissue obtained from controls (left column) and from cyclosporine A-treated animals (right column). CsA, cyclosporine A.

cyclosporine A-induced apoptosis. Cyclosporine A promoted a caspase-independent release of cytochrome *c* and of second mitochondrial activator of caspases/direct inhibitor of apoptosis protein binding protein with low pI (Smac/Diablo) from mitochondria. Cyclosporine A also led to a caspase-dependent loss of mitochondrial membrane potential. Caspase 2, Caspase 3 and Caspase 9 were activated, and specific caspase inhibitor prevented apoptosis and increased long-term survival.

Recently, we reported that cyclosporine A up-regulated the expression of p21 in gingival epithelial cells and increased the stasis of cells in the G1/G0 phase (7). Strong evidence suggests that p21 is one of the most important downstream target genes of p53, a tumor suppressor gene (8); however, studies have also shown that p21 may be induced by p53-independent pathways (32). Enhanced expression of transforming growth factor beta and of the transforming growth factor beta-1 receptor in cyclosporine A-induced gingival overgrowth has recently been observed (33,34). Possible functions of transforming growth factor beta-1 in cyclosporine A-induced overgrown gingivae have been explored, including fibroblast proliferation (35), angiogenesis (33), tissue fibrosis (36), inhibition of matrix metalloproteinases and matrix protein accumulation (37). An *in vitro* study has indicated that transforming growth factor beta-1 induces p21 expression and apoptosis in a transforming growth factor beta-1-dependent manner (38). Enhanced expression of epidermal growth factor (EGF) and of its receptor after treatment with cyclosporine A was observed in edentulous gingivae of rats and in OECM1 cells in our laboratory (6). EGF can enhance cell proliferation and DNA synthesis and has been thought to be a mitogen for fibroblasts and for epithelial cells (39). By contrast, long-term treatment with EGF may suppress cell growth, induce apoptosis and enhance p21 expression in the A431 squamous carcinoma cell line that overexpresses the EGF receptor (40,41). Thus, long-term elevated levels of transforming growth factor beta-1 and EGF in gingival tissue during cyclosporine A therapy may indirectly contribute to the apoptosis of keratinocytes.

In the present *in vivo* experiment, the edentulous gingival tissue from the alveolar ridge of rat was selected because such local anatomy has no gingivo-tooth interface, little bacterial plaque accumulation, but would still have significant overgrowth (4). Studies have shown that periodontal pathogens, including *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, can induce the apoptosis of epithelial cells (42–45). The effects of *Treponema denticola* on extracellular signal-regulated kinase (ERK), p38 and Jun N-terminal kinase mitogen-activated protein (MAP) kinases, and on cell behavior, were studied using nonkeratinizing periodontal ligament epithelial cells *in vitro*. Terminal transferase dUTP nick end labeling (TUNEL) staining analysis showed that about 50% of epithelial cells in monolayers died as a result of apoptosis when exposed to a high dose of *T. denticola* for

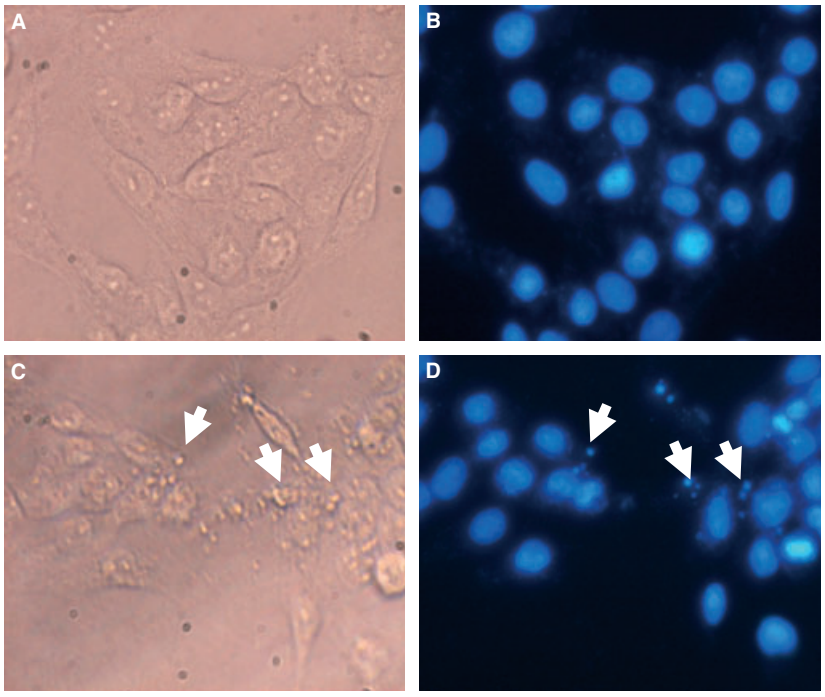
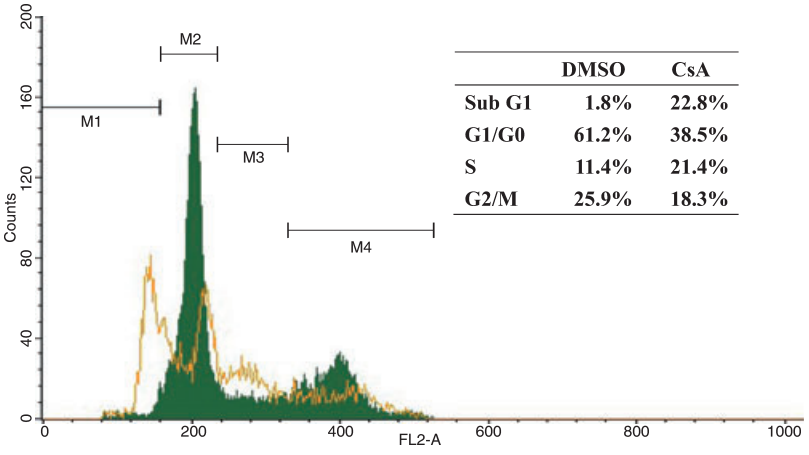


Fig. 4. Formation of apoptotic bodies in OECM1 cells after treatment with cyclosporin A (1000 ng/mL). Microphotographs show the morphology of OECM1 cells with or without 24 h of treatment with cyclosporine A. After staining with 4′,6-diamidino-2-phenylindole (DAPI), the apoptotic bodies were easily observed in cells treated with cyclosporine A. (A, B) Cells treated with solvent. (C, D) Cells treated with cyclosporine A. (A, C) Analysis using phase-contrast microscopy. (B, D) Analysis after DAPI staining. Arrows indicate apoptotic bodies. Original magnification ×150.



	DMSO	CsA
Sub G1	1.8%	22.8%
G1/G0	61.2%	38.5%
S	11.4%	21.4%
G2/M	25.9%	18.3%

Fig. 5. Cell cycle phase distributions of OECM1 cells after 32 h of treatment with dimethylsulfoxide (DMSO) (green) or with 1000 ng/mL of cyclosporine A (yellow), as expressed by flow cytometry. (Sub-G1 phase, M1; G0/G1 phase, M2, S phase, M3; and G_2/M phase, M4). CsA, cyclosporine A.

24 h. Western blot analysis using MAP kinase-phosphospecific antibodies showed that *T. denticola* strongly, but transiently, activated ERK1 and ERK2 (signals mediating cell proliferation) and JNK and p38 (kinases mediating apoptosis). While a specific inhibitor of the ERK MAP kinase pathway prevented the *T. denticola* stimulation of cell proliferation, inhibitor of p38 increased the cell numbers in *T. denticola*-treated cultures (46).

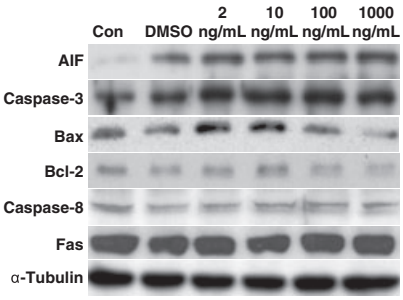


Fig. 6. Western blot analysis of the expression of apoptosis-inducing factor (AIF), Caspase 3, bcl2-associated X protein (Bax), Bcl-2, Caspase 8 and Fas proteins in OECM1 cells in the control and following incubation with dimethylsufoxide (DMSO) alone, or with different concentrations of cyclosporine A (2, 10, 100 and 1000 ng/mL in DMSO). (The experiments were repeated three times.) Con, control.

In the present *in vitro* study, the cells of OECM1, a human gingival squamous carcinoma cell line, but not the keratinocytes of healthy gingiva, were used. The human papilloma virus-immortalized normal human oral keratinocyte cell line (HOK-16B), the epitheloid cervical carcinoma cell line (HeLa) and primary oral keratinocytes have been previously used to test *in vitro* the hypothesis of cyclosporine A-influenced growth and apoptosis of oral epithelial cells (28). The results of this previous study showed that cyclosporine A inhibited cell division in all three types of keratinocyte cells, but cyclosporine A (1 μg/mL) did not have any effect on constitutive or tumor necosis factor-alfa-induced apoptosis, or on Bcl-2 expression, in HOK-16B cells or in cells of the human cervix epitheloid cell line. In the present study, however, 1 μg/mL of cyclosporine A could induce the formation of apoptotic bodies and increase the number of OECM1 cells in the sub-G1 phase. In OECM1 cells, our data also showed enhanced expression of AIF and Caspase 3 occurred at cyclosporine A concentrations of greater than 2 ng/mL and enhanced expression of Bax occurred at cyclosporine A concentrations of 2, 10 and 100 ng/mL.

In conclusion, this study tested the hypothesis that cyclosporine A treatment leads to the apoptosis of gingival

keratinocytes. Cell apoptosis was initially suggested because *in vivo* cyclosporine A altered the ratios of certain apoptotic genes and increased the number of DNA fragments and *in vitro* enhanced the number of OECM1 cells exhibiting DAPI-stained apoptotic bodies and the number of OECM1 cells in the sub-G1 phase. Furthermore, it was suggested that the signals of apoptosis were associated with to the mitochondrial pathway because, both in *in vivo* and *in vitro* experiments, the expression of signals was stronger in the mitochondrial pathway (Bax and AIF) and in the common pathway (Caspase 3), than in the death receptor pathway (Fas and Caspase 8), after treatment with cyclosporine A. Therefore, we suggest that cyclosporine A can enhance cell apoptosis, via the Bax-mediated mitochondrial pathway, in rat gingival keratinocytes and in OECM1 cells. The enhanced apoptosis in epithelia, in combination with the observed epithelial proliferation in our previous studies (6,47), might also suggest a rapid turnover of keratinocytes in gingiva during treatment with cyclosporine A.

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