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Expression of p21 and p53 in rat gingival and human oral epithelial cells after cyclosporine A treatment

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Background and Objective: Expression of p21 and p53 were examined, at gene and protein levels, in edentulous gingival epithelial cells from rats and from a human oral epidermoid carcinoma cell line, OECM1, after cyclosporine A therapy.

Material and Methods: In vivo: 20 partially edentulous SD rats were assigned into cyclosporine A feeding and control groups. After the rats were killed, p21 and p53 in gingiva were evaluated by reverse transcription-polymerase chain reaction and immunohistochemistry. *In vitro:* after cyclosporine A treatment, p21 and p53 of OECM1 cells were evaluated by western blot and the luciferase assay. The distribution of OECM1 cells in each phase of the cell cycle was evaluated by flow cytometry.

Results: The mRNA expression of p21 was significantly higher in the cyclosporine A group than in the control group. A greater number of positive anti-p21-stained cells were observed in the gingival epithelium of the cyclosporine A group than in the control group. Significantly higher levels of p21 protein and activity were observed in OECM1 cells after cyclosporine A treatment than in cells without treatment. A relative increase of cells in G0/G1 phases, and a decrease of cells in G2/M phases, were observed in OECM1 cells after cyclosporine A treatment.

Conclusion: In the present study, higher p21 mRNA and protein expressions were observed after cyclosporine A treatment. Thus, an up-regulation of p21 expression, via a p53-independent pathway, by cyclosporine A in gingival and oral epithelial cells was suggested.

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Systemic cyclosporine A administration can induce gingival overgrowth (1–4). This effect may be related to cyclosporine A-induced epithelial hyperplasia (5–7). Studies have found that cyclosporine A up-regulates epithelial growth factor and keratinocyte growth factor (8–10), both of which are produced by epithelial cells. The detailed mechanisms of drug-elicited gingival epithelial hyperplasia, in particular drug effects on cell cycle regulation, are still unknown.

It was proposed that transactivation of p21 might halt the cell cycle through the cyclosporine A-induced accumulation of p53, because cell cycle arrest induced by cyclosporine A was coincident with elevated levels of p53 in renal tubular epithelial cells (11). When neutralizing antibody to transforming growth factor-β was used to affect transforming growth factor-β production, the induction of p21 by cyclosporine A was inhibited (12). In addition, p53-independent and p53-dependent induction of p21 mRNA occurred simultaneously in murine kidney cells (13). Whether p21 has any involvement in cyclosporine A-induced gingival epithelial hyperplasia (via either the p53-dependent or the p53-independent pathway) has never been

evaluated. In this study, the gene and protein expression of p21 after cyclosporine A therapy was examined in the overgrown edentulous gingivae of rats (7) and in an oral epithelial cell line (OECM1) (10), derived from a human oral epidermoid carcinoma, to elucidate the roles of p21 and p53 in cyclosporine A-induced gingival epithelial alterations.

Material and methods

In vivo experiment

Twenty male 5-wk-old Sprague–Dawley rats, weighing 120–150 g, were used in the study. The rats were randomly assigned into a cyclosporine A group and a control group after a 3-wk wound-healing period following extraction of all the maxillary right molars, as in our previous study (7). 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 rats in the control group received mineral oil alone. At the end of the study, all animals were killed by carbon dioxide inhalation. Half of the edentulous gingival specimens (five from each group) were immediately frozen in liquid nitrogen and stored at −70°C for later evaluation by reverse transcription–polymerase chain reaction (RT–PCR). The other specimens were immediately fixed in 4% (v/v) paraformaldehyde. After paraffin embedding, serial tissue sections, 4 μm thick, were sliced buccopalatally and examined by immunohistochemistry to evaluate the expression of p21 and p53 proteins.

In vitro study

The oral epithelial cell line, OECM1, derived from a human oral epidermoid carcinoma, was a generous gift from Dr Ching-Liang Meng at the National Defense Medical Center. Cells were grown in RPMI-1640 medium (GibcoBRL Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) 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) (10). The cells were cultured in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO_2 at 37°C for 16 h. The cells were rinsed with sterile phosphate-buffered saline before cyclosporine A treatment, and then stimulated using 1% (v/v) fetal bovine serum culture medium supplemented with different concentrations (0, 10^2, 10^3 or 10^4 ng/mL) of cyclosporine A in dimethylsulfoxide (Sigma Chemical Co., St Louis, MO, USA) (14). During the first 2 d of cyclosporine A treatment, cells were harvested at 0, 6, 12, 18, 24 or 48 h to evaluate cell cycle arrest by flow cytometry and to examine p21 expression and activity by western blotting and with a luciferase assay, respectively.

Immunohistochemistry (*in vivo*)

After removal of paraffin, and hydration, tissue sections were boiled in DAKO buffer (Dako Cytomation, San Diego, CA, USA) for 15 min for antigen retrieval. Endogenous peroxidase activity was quenched by incubation for 5 min with 0.1% (v/v) hydrogen peroxide in distilled water. Tissue sections were washed twice, for 5 min each wash, in phosphate-buffered saline and incubated for 2 h with non-conjugated primary polyclonal antibodies against p21 and p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (15,16), followed by further incubation with biotinylated secondary antibody, streptavidin-conjugated horseradish peroxidase complexes and 3-amino-9-ethyl carbazole solution (Dako Cytomation) for further periods of 30 min, 30 min and 10 min, respectively. Between incubations, cells and sections were washed with sterile phosphate-buffered saline. Specimens were then counterstained with hematoxylin, dehydrated and mounted. Cells showing positive staining for p21 and p53 were examined under a microscope.

RNA extraction and RT–PCR (*in vivo and in vitro*)

Gingival tissue and OECM1 cells were homogenized. Total RNA was extracted with Trizol and quantified by spectrophotometry at 260 nm. The PCR system used was the GeneAmp®-9700 kit (Applied Biosystems, Foster City, CA, USA). Initially, 5 μg of total RNA was reverse-transcribed into total cDNA at 55°C for 1 h and this material was used as a template for PCR reactions and analysis. The PCR reactions involved an initial denaturation at 94°C for 2.5 min, followed by 30 or 35 cycles at 94°C for 30 s, annealing at 58–62°C for 30 s, and polymerization at 72°C for 60 s. The PCR primer sequences used to amplify p21 and p53 sequences are shown in Table 1 (17). The number of RT–PCR cycles was either 30 or 35 to allow quantitative comparison of the cDNAs. Amplified RT–PCR products were run on 1% (w/v) agarose gels, stained with ethidium bromide and photographed (Transilluminator/SPOT Diagnostic Instruments, Sterling Heights, MI, USA). Gel images of RT–PCR products were directly scanned (ONE-DSCAN 1-D Gel Analysis Software; Scanalytic Inc., Fairfax, VA, USA) and relative densities were obtained by determining the ratio of signal intensities to glyceraldehyde-3-phosphate dehydrogenase bands (18).

Table 1. The primers used to amplify p53, p21 and glyceraldehyde-3-phosphate dehydrogenase (a housekeeping gene), and the expected polymerase chain reaction product sizes

Gene	Primer sequence		Product size (bp)	Reference
Rat p53	Sense	CACAGTCGGATATGAGCATC	600	(17)
	Antisense	GTCGTCCAGATACTCAGCAT		
Rat p21	Sense	GTGAGACACCAGAGTGCAAGA	400	(17)
	Antisense	ACAGCGATATCGAGACACTCA		
Rat GAPDH	Sense	TGCTGGTGCTGAGTATGTCG	646	(18)
	Antisense	ATTGAGAGCAATGCCAGCC		

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Gene expression in test (i.e. cyclosporine A treated) and control groups was compared.

Flow cytometry of OECM1 cells upon cyclosporine A therapy (*in vitro*)

One milliliter of cell suspension was diluted to 10 mL with phosphate-buffered saline and centrifuged at 300 ***g*** for 5 min at 4°C. The cell pellet was resuspended in 1 mL of phosphate-buffered saline, fixed in absolute ethanol at −20°C and stored at −20°C for subsequent flow cytometry analysis. Before flow cytometry, the cell suspension was centrifuged and resuspended in 1 mL of phosphate-buffered saline. A 100-μL volume of RNase A (Sigma Chemical Co.) (200 μg/mL), boiled to eliminate DNase, was added and the suspension was incubated at 37°C for 30 min. One-hundred microlitres 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 ten-fold in phosphate-buffered saline immediately prior to 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) (19).

Luciferase assay for p21 and p53 activities (*in vitro*)

OECM1 cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum. A total of 5×10^4 OECM1 cells were seeded into 24-well plates and incubated overnight. Transient transfections were performed using the FuGene reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. The p21-LUC (20) (0.6 μg) and the pG13-LUC (21) (0.6 μg) reporter plasmids (kindly provided by Dr S. M. Huang) (22) were used to transfect the p21 and p53 genes, respectively. To evaluate p53 activities in OECM1 cells (23,24), a histone deacetylase inhibitor, trichrostatin A (0.25 ng/mL) (Sigma Chemical Co.), was used. Luciferase activities of wild-type p53-transfected and trichrostatin A-treated cells were measured using the Luciferase Assay System (Promega Corp., Madison, WI, USA) with a luminometer (Lumat LB9501; Berthold GmbH & Co. KG, Bad Wildbad, Germany) as described in the manufacturer's manual, and data are presented as relative light units, expressed as means and standard deviations of data from three transfected cultures (25).

Western blotting of p53 and p21 (*in vitro*)

Homogenates of lysed OECM1 cells were centrifuged (13,000 *g*, 4°C, 15 min) and then boiled. Protein concentrations were determined by a protein microassay, using the BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL, USA), and proteins were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 18% (w/v) polyacrylamide gels. Proteins were electroblotted to poly(vinylidene difluoride) membranes. Nonspecific binding was blocked by incubating blots for 1 h in 10% (v/v) fetal calf serum. After six washes with phosphate-buffered saline containing 0.005% (v/v) Tween 20, the p21, p53 and actin proteins were detected by incubation with primary antibodies (1 : 1000 dilutions of mouse monoclonal anti-p21, rabbit polyclonal anti-p53 and mouse monoclonal anti-actin; Chemicon International Inc., Temecula, CA, USA) (15,16,26) overnight at 4°C followed by incubation with secondary antibody (goat antimouse or goat antirabbit) (Santa Cruz Biotechnology), conjugated with IgG-horseradish peroxidase at a dilution of 1 : 5000, for 1 h. Antibody-reactive proteins were detected using enhanced chemiluminescence. Optical densities were obtained after three determinations for each band in three different gels. The expressions of p21 and p53 in cyclosporine A-treated OECM1 cells are presented as proportional increases or deceases relative to the levels observed in control cells. In this study, cells treated with 10% (v/v) fetal bovine serum culture medium and dimethylsulfoxide solvent are controls, whereas cells treated with 100 and 1000 ng/mL of cyclosporine A are experimental groups. After 24 h of treatment, cells of all groups were harvested for western blotting of proteins.

Statistical analysis

Student's *t*-tests were used to evaluate differences between the control group and the cyclosporine A group in the expression of mRNAs of p21, p53 and glyceraldehyde-3-phosphate dehydrogenase (relative densities by RT–PCR) and the distribution of OECM1 cells in each phase of the cell cycle, as measured with flow cytometry. A p-value of < 0.05 was selected as the significance level.

Results

In vivo study

The expression of p21 mRNA, as measured by RT–PCR, was significantly higher in gingival tissues of cyclosporine A-treated animals than in gingival tissues of the control group, whereas p53 expression was similar in the two groups (Fig. 1). Immunohist-

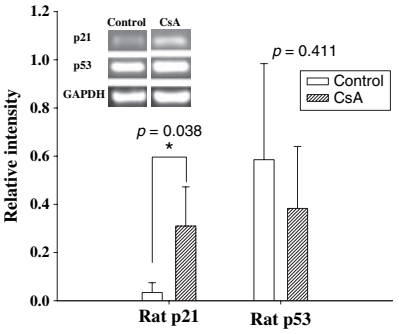

Fig. 1. Comparison of p21 and p53 mRNA expression levels (relative to that of glyceraldehyde-3-phosphate dehydrogenase mRNA) in rat edentulous gingival tissue. A test group (exposed to cyclosporine A) (hatched bars) and a control group (white bars) are compared. Means and standard deviations are given, and significant differences at $p < 0.05$ are marked with asterisks. The insert is a gel image showing mRNA encoding p21, p53 and glyceraldehyde-3-phosphate dehydrogenase extracted from gingival tissue of a control and a cyclosporine A-treated rat. CsA, cyclosporine A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

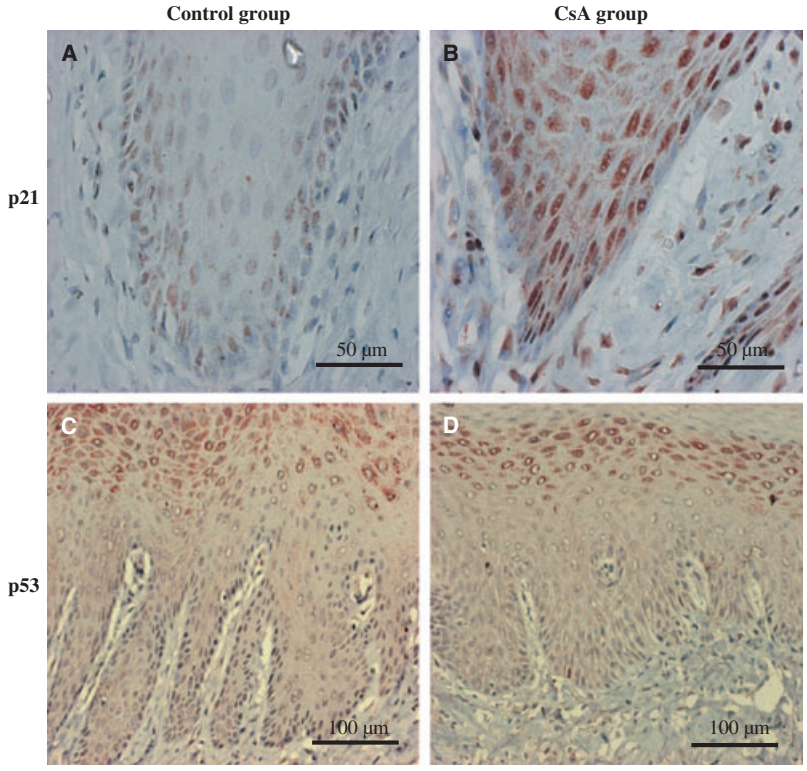

Fig. 2. Micrographs showing immunohistochemical staining of p21 and p53 in gingival tissue from control and cyclosporine A-treated rats. (A) p21 control; (B) p21 cyclosporine A-treated; (C) p53 control; and (D) p53 cyclosporine A-treated. CsA, cyclosporine A.

ochemistry showed that many cells of gingival tissues from the control and cyclosporine A-treated animals stained positive for p21 and p53. Such cells included both epithelial and connective tissue stromal cells. A greater number of positively staining p21-expressing cells were observed on the gingival epithelial layer in the cyclosporine A group than in the control group, but the numbers of cells with p53 staining were similar in the two groups (Fig. 2).

In vitro study

Western blotting results showed more p21 protein in OECM1 cells treated with cyclosporine A (100 and 1000 ng/mL) than in cells without cyclosporine A treatment (including cells receiving no treatment or dimethylsulfoxide solvent treatment only) (Fig. 3). Activities of the p21 and p53 promoters were evaluated by luciferase assays (Fig. 4). Detectable levels of activity of the p21 promoter (from the p21-LUC plasmid) were recorded for up to 48 h after

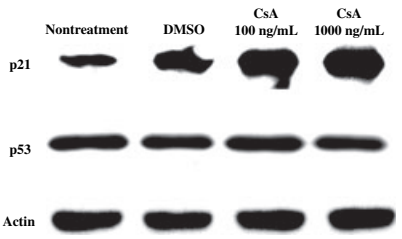

Fig. 3. Effect of cyclosporine A on the expression of p21, p53 and actin proteins, as shown by western blotting, in OECM1 cells after treatment with dimethylsulfoxide (solvent) or cyclosporine A at either 100 or 1000 ng/mL. The experiment was repeated three times. CsA, cyclosporine A; DMSO, dimethylsulfoxide.

treatment of the cells with 10^3 ng/mL of cyclosporine A (Fig. 4A). Higher p21 promoter activities were observed in cells after cyclosporine A treatment than in cells without cyclosporine A exposure (Fig. 4B). The highest activities were recorded when cyclosporine A was used at 10 ng/mL and 10^2 ng/mL. Detectable p53 promoter activities were recorded in OECM1 cells after p53 transfection and trichrostatin A treatment (Fig. 4C) but p53 promoter activities were quite low in OECM1 cells after cyclosporine A treatment. Flow cytometry showed that the cell cycle distributions of OECM1 cells changed when cells received cyclosporine A treatment (Fig. 5). An increase in the proportion of cells in the G0/G1 phases, and a corresponding decrease in cells in the G2/M phases, was observed in cells exposed to cyclosporine A when compared to cells without cyclosporine A (Table 2).

Discussion

In this study, the gene and protein expressions of p21 and p53 upon cyclosporine A therapy were examined in edentulous gingivae of rats and in the human epithelial cell line, OECM1. Protein p21, also called WAF1, CAP20, Cip1, or Sdi1 (21,27–29), is the founding member of the Cip/Kip family of cyclin-dependent kinase inhibitors, which also includes p27 (30,31) and p57 (32,33). The cyclin-dependent kinase inhibitors bind to, and inhibit, a broad range of cyclin/cyclin-dependent kinase complexes (34,35). In normal cells, cyclin-dependent kinases exist predominantly in multiple quaternary complexes, each containing a cyclin-dependent kinase, cyclin, the proliferating cell nuclear antigen and the p21 protein, whereas proliferating cell nuclear antigen and p21 are lost in some transformed cells (28). Protein p21 plays an essential role in growth arrest after DNA damage (36–38), and overexpression of p21 leads to G1 and G2 (39) or S-phase arrest (40). In addition to regulating normal cell cycle progression, p21 integrates genotoxic signal insults into apoptotic signaling pathways that ultimately determine cell fate (41).

The tumor suppressor gene, p53, plays a fundamental role in controlling cell cycle checkpoints, apoptosis and genetic stability. Wild-type p53 controls these processes by regulating the transcription of target genes through binding to consensus DNA sites in promoter regions (42). Transcriptional targets of p53 have been identified and regulate DNA repair, cell growth, or cell death processes (43–50). Strong

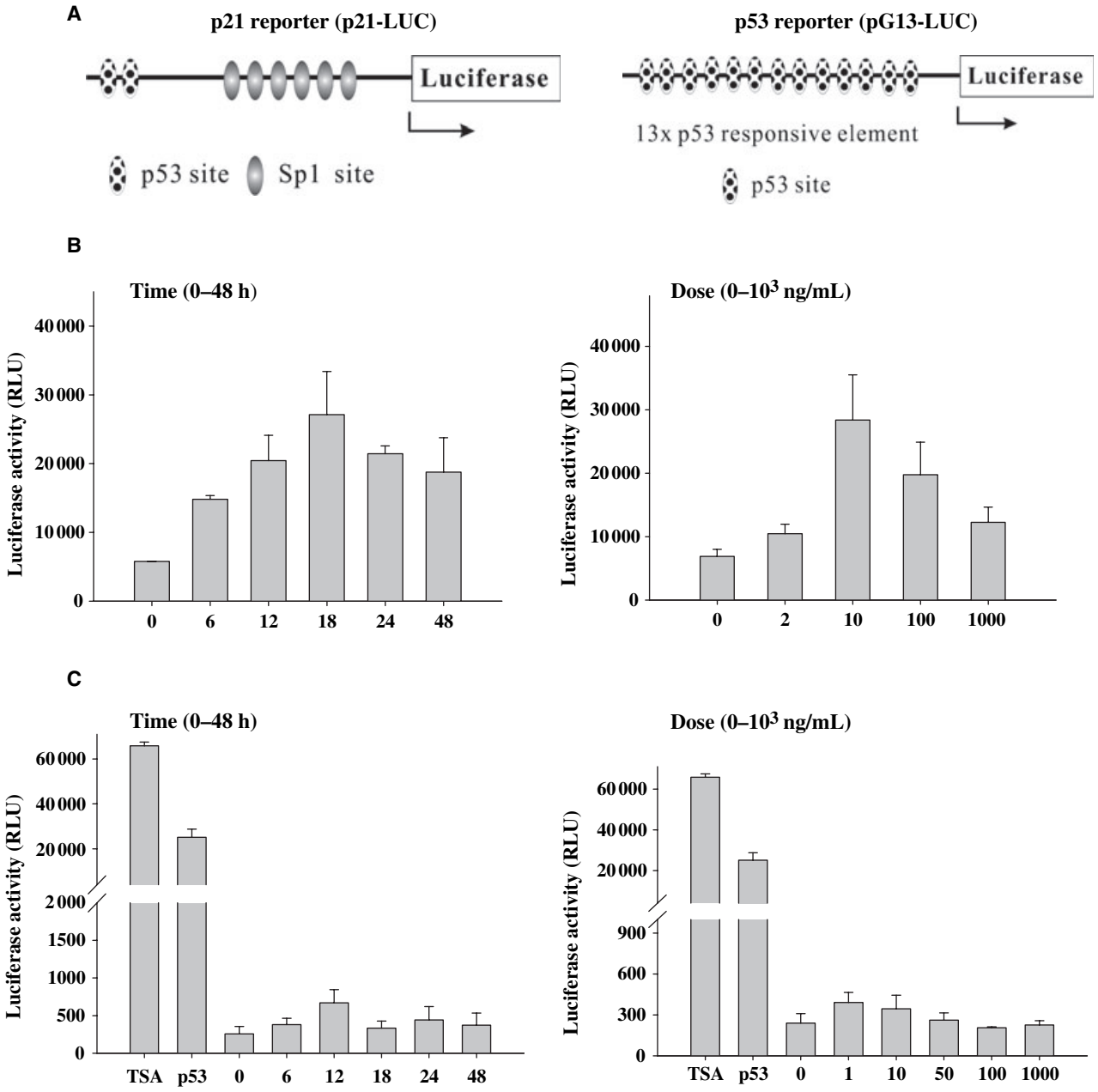

Fig. 4. Activities of the p21 and p53 promoters, using a luciferase detection assay, in OECM1 cells after cyclosporine A treatment. (A) The scheme of p21 and p53 reports. (B,C) The luciferase activities for p21 and p53, respectively. The activities were measured at 0, 6, 12, 18, 24 and 48 h after stimulation with cyclosporine A at 10^3 ng/mL. The dose-dependent study used cyclosporine A at concentrations up to 10^3 ng/mL and a 24-h exposure time. The means and standard deviations of three experiments are shown. RLU, relative light units; TSA, trichrostatin A.

evidence suggests that one of the most important downstream target genes of p53 is p21 (28). p21 is directly induced by wild-type p53 through consensus p53-binding sequences in the p21 promoter (21). Some studies have also shown, however, that p21 may be induced by p53-independent pathways (51–53). In the present study, cyclosporine A induced a greater expression and activity of p21, but not of p53, both *in vivo and in vitro*. We therefore suggest that cyclosporine A may up-regulate p21 expression via a p53-independent pathway in gingival epithelial cells. The up-regulated p21 may act independently of p53 to increase cell stasis in the G0/G1 phases.

Enhanced expression of transforming growth factor-β and of the transforming growth factor-β receptor in cyclosporine A-induced gingival overgrowth has recently been observed (54,55). Possible functions of transforming growth factor-β1 in cyclosporine A-induced overgrown gingivae have been explored. Such functions may include fibroblast proliferation (56), angiogenesis (54), tissue fibrosis (57), inhibition of matrix metalloproteinases

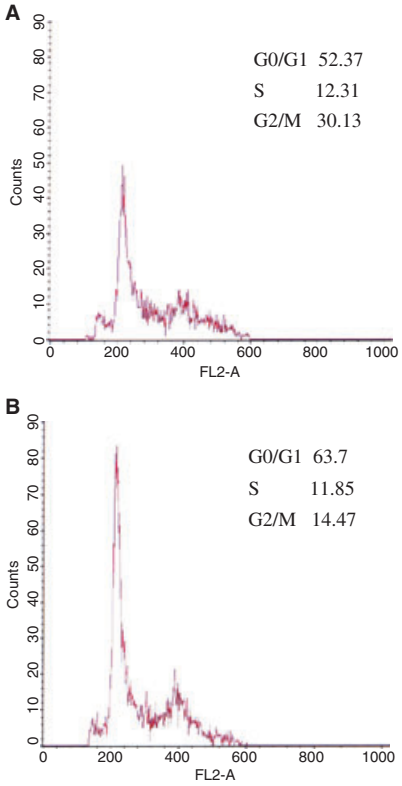

Fig. 5. Cell cycle phase distributions of OECM1 cells after treatment with dimethylsulfoxide solvent (A) or 1000 ng/mL of cyclosporine A (B) for 24 h. CsA, cyclosporine A; DMSO, dimethylsulfoxide.

and matrix protein accumulation (58). An *in vitro* study has further indicated that transforming growth factor-β1 induces p21 expression and apoptosis in a transforming growth factor-β1-dependent manner (12). The prominent expression of p21 shown in the present study may occur partially because of transforming growth factor-β1 elevation.

Enhanced expression of epithelial growth factor and its receptor after cyclosporine A treatment were observed in the edentulous gingivae of rats and OECM1 cells in our previous study (10). Epithelial growth factor can enhance cell proliferation and DNA synthesis and has been thought to be a mitogen for fibroblasts and epithelial cells (59,60). In contrast, long-term treatment with epithelial growth factor may suppress cell growth, induce apoptosis and enhance p21 expression in the A431 squamous carcinoma cell line that overexpresses the epithelial growth factor receptor (61–63). Thus, long-term elevated levels of epithelial growth factor and the epithelial growth factor receptor in gingival tissue during cyclosporine A therapy may, in part, contribute to the increased expression of p21 seen here.

In the present work, the expression of p53 was not affected by cyclosporine A therapy, either *in vivo* or *in vitro*. In the *in vitro* study, OECM1 cells, and not normal gingival epithelial cells, were used. To measure p53 activities in transformed OECM1 cells, activities were recorded after transfection of wild-type p53 and trichrostatin A treatment (Fig. 4C). Studies have shown that trichrostatin A can stimulate wild-type p53 activities in both normal and transformed cells (23,24,64). In addition, escaping from cell cycle arrest and apoptosis may be a mechanism related to cell immortalization (65,66), as shown by the OECM1 cell line. The elevated p21 expression during cyclosporine A therapy in this study may indicate cell cycle arrest or further apoptosis in the immortal OECM1 cells.

In conclusion, the present *in vitro* and *in vivo* studies show that cyclosporine A therapy leads to the overexpression of p21, but not of p53. Treatment with cyclosporine A caused an increased distribution of OECM1 cells in the G0/G1 phases and a corresponding decrease of cells in the G2/M phase. Therefore, we suggest that cyclosporine A may up-regulate p21 expression by a p53-independent pathway, leading to epithelial cell cycle stasis in oral/gingival cells. Because of the limitations of our study, the p53-dependent up-regulation of p21 expression is still not completely neglected. In addition, the present discovery of cyclosporine A-induced cell cycle stasis, combined with the enhanced cell proliferation reported in our previous studies, may further indicate a rapid cell turnover in cyclosporine A-induced gingival overgrowth.

Table 2. Cell cycle phase distributions in OECM1 cells exposed to 10^3 ng/mL of cyclosporine A or dimethylsulfoxide control

Cell population	Dimethylsulfoxide	Cyclosporine A	*p*-value
Sub-G1 (%)	2.84 ± 0.61	6.04 ± 1.95	0.135
G0/G1 (%)	58.53 ± 2.96	67.72 ± 3.08	0.045*
S (%)	13.13 ± 0.94	12.14 ± 1.09	0.499
G2/M (%)	23.80 ± 2.99	13.66 ± 2.88	0.025*

The figures are percentages; means and standard deviations are shown.
Sub-G1 phase, < 2 N DNA; G1 phase, 2 N DNA; S phase, > 2 N and < 4 N DNA; and G_2/M phase, 4 N DNA.
*Significantly different at $p < 0.05$.

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