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Up-regulation of retinoblastoma protein phosphorylation in gingiva after cyclosporine A treatment: an *in vivo* and *in vitro* study

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Background and Objective: Cyclosporine A can induce gingival cell proliferation; however, the precise molecular regulation of the proliferation is uncertain. Therefore, this study was carried out to examine, *in vivo* and *in vitro*, the expression of genes and proteins associated with gingival cell proliferation after treatment with cyclosporine A.

Material and Methods: Forty Sprague Dawley rats with right maxillary posterior edentulous gingivae were assigned to a cyclosporine A group (30 mg/kg daily of cyclosporine A, administered orally) or a control group (administered mineral oil only). The animals were killed 4 wk after treatment. The edentulous gingivae were dissected out and analyzed for the expression of proliferating cell nuclear antigen (PCNA), cyclin D1, cyclin-dependent kinase 4 (CDK4) and retinoblastoma protein (Rb1) mRNA and/or protein, and phosphorylated Rb1 (pRb1), by real-time RT–PCR or immunohistochemistry. In human gingival fibroblast (HGF) cultures, the expression of PCNA, CDK4, cyclin D1 and Rb1 proteins and Rb1 phosphorylation were determined by western blotting after cyclosporine A treatment ($0–10^4$ ng/mL).

Results: Proliferating cell nuclear antigen and cyclin D1 mRNAs (*Pcna* and *Ccnd1*, respectively) were expressed more strongly in the gingivae of cyclosporine A-treated animals than in the gingivae of the controls. Immunohistochemical analyses showed that a greater number of gingival cells stained positive for cyclin D1, CDK4 and pRb1 in the cyclosporine A group than in the control group. Increased expression of cyclin D1, CDK4 and PCNA proteins was observed in HGFs after cyclosporine A treatment. The phosphorylation of Rb1 was enhanced in HGFs after treatment with cyclosporine A at concentrations of $10^2–10^3$ ng/mL.

Conclusion: The increases in cyclin D1, PCNA and CDK4, together with the enhanced phosphorylation of Rb1, suggest that cyclosporine A promotes cell-cycle progression through the G_1/S transition in the gingiva.

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Systemic cyclosporine A administration induces gingival overgrowth, which is characterized by the overproduction of extracellular matrix components, a large number of fibroblasts and epithelial thickening (1). The direct and indirect effects of the drug on gingival fibroblasts and their metabolism have previously been examined (2–4). Cyclosporine A treatment can induce the proliferation of gingival fibroblasts (1,3,5–8), but the precise molecular regulation of cyclosporine A-stimulated gingival cell proliferation is not yet completely clear.

The progression of the cell cycle is governed by a family of cyclin-dependent kinases (CDKs) and their regulatory subunits, the cyclins (9–11). A key role of the CDKs is to inactivate, by phosphorylation, the negative regulators of cell cycle progression, notably retinoblastoma protein (Rb1), to permit cell exit from G_1 and entry into the S phase (12,13). Proliferating cell nuclear antigen (PCNA) is a protein that functions as an auxiliary protein for DNA polymerase d (14,15). PCNA is expressed at the start of the late G_1 stage. Its expression increases two- to three-fold in the S phase, and decreases at the S/G_2 transition and during G_2/M.

Studies have shown a precise correlation between the proliferative state of the cell and the stage of the cell cycle: increasing through G_1, peaking at the G_1/S phase transition, decreasing through G_2 and reaching low levels at the G_2/M transition and in M phase (16,17). Checkpoints are now known to exist at every single point in the cell cycle. The first checkpoint, called the 'restriction point', is located at the end of the G_1 phase of the cell cycle, just before entry into the S phase. The restriction point is predominantly controlled by the action of the CDK inhibitor, p16. This protein inhibits CDK4/6 and ensures that they can no longer interact with cyclin D1 to cause cell cycle progression. Once the CDK4/6–cyclin D complex is activated, it phosphorylates Rb1, which results in its dissociation from E2F. E2F is then able to induce the expression of cyclin E, which interacts with CDK2 to allow the G_1/S phase transition (18). These cyclin–CDK complexes induce the phosphorylation of Rb1, and phosphorylated Rb1 (pRb1) promotes cell cycle progression through the G_1/S transition (19,20). Because the precise molecular regulation of cyclosporine A-stimulated gingival cell proliferation remains unclear, the expression of genes or proteins associated with its proliferation, such as PCNA, cyclin D1, CDK4 and Rb1, and the phosphorylation of Rb1, were investigated *in vivo* in a rat model and *in vitro* in primary cultured human gingival fibroblasts (HGFs) after their treatment with cyclosporine A.

Material and methods

In vivo experiment

Forty, male, 5-wk-old Sprague Dawley rats, weighing 120–150 g, were randomly assigned to a cyclosporine A group or a control group 3 wk after the extraction of all their right maxillary molars (21). Animals in the cyclosporine A group received cyclosporine A (30 mg/kg body weight in mineral oil; Sandimmun, Sandoz, Basel, Switzerland) daily by gastric feeding for 4 wk, whereas the control-group rats received mineral oil only. At the end of the study, all animals were killed with carbon dioxide inhalation. Ten edentulous gingival specimens from each group were immediately frozen in liquid nitrogen and stored at −70°C for the real-time RT–PCR assay of genes related to cell proliferation, including *Pcna* and *Ccnd1*. After paraffin embedding, serial sections of tissue were cut buccopalatally at a thickness of 4 μm, and analyzed using immunohistochemistry (IHC) to evaluate the expression of cyclin D1, CDK4 and pRb1 proteins (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Real-time RT–PCR assay

Total RNA from homogenized gingival tissue was extracted using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA, USA) and quantified by spectrophotometry at 260 nm. On a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA), 5 μg of each total RNA was reverse transcribed with SuperScript III at 55°C for 1 h into total complementary DNA, which was used as the template for the subsequent PCR reactions and analysis. Confirmation of *Pcna* and *Ccnd1* gene expression was confirmed using the ABI Real-time PCR System (22). In brief, the desired probes and primers for rat *Pcna*, *Ccnd1* and β-actin (*Actb*) were selected from TaqMan Assay-on-Demand gene expression. 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. Each primer/probe set (5–10 μL) was then added and the PCR was conducted with the following cycling parameters: one cycle at 95°C for 12 min and 40 cycles of 95°C for 20 s and 60°C for 1 min. The data were analyzed using sequence detection software that calculates the threshold cycle for each reaction (23).

Immunohistochemistry

After deparaffinization and hydration, the tissue sections were boiled in Dako buffer (Dako Denmark, Glostrup, Denmark) for 15 min to facilitate antigen retrieval. The endogenous peroxidase activity was quenched by incubation for 5 min with 0.1% hydrogen peroxide in distilled water. The sections were then incubated for 2 h with unconjugated primary polyclonal antibodies directed against CDK4, cyclin D1 or pRb1. This was followed by incubation with biotinylated secondary antibody, streptavidin-conjugated horseradish peroxidase complexes and 3-amino-9-ethyl carbazole solution for a further 30, 30 and 10 min, respectively. The specimens were then counterstained with hematoxylin, dehydrated and mounted. Cells showing positive staining for CDK4, cyclin D1 and pRb1 were identified by microscopy with 100X magnification (BX50, Olympus, Japan). In this study, five consecutive tissue sections were selected from each animal to determine and compare the percentages of positively stained cells, obtained after immunohistochemical preparation, in the gingival stroma of the control and cyclosporine A-treated rats (22,24).

In vitro experiment

As in our previous study, the connective tissue fragments of gingiva were digested for 24 h in medium containing 10% fetal bovine serum (FBS) and 2 mg/mL of collagenase (Sigma-Aldrich Inc., St Louis, MO, USA) (24). The fragments were then placed in culture flasks to allow the cells to migrate from the explants, and the fibroblast cultures were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 10% FBS. After stimulation, for 24 h, with various concentrations of cyclosporine A (1, 10, 10^2, 10^3 and 10^4 ng/mL) prepared in dimethylsulfoxide (DMSO, a solvent) the cells were harvested. Their proliferation was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA), and the expression of PCNA, CDK4, cyclin D1 and Rb1 proteins, and the phosphorylation of Rb1, was evaluated by western blotting.

Proliferation assay

HGFs were cultured in DMEM supplemented with 2 mmol/L of L-glutamine and 10% FBS. Cell viability was analyzed using the MTS assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega). In brief, HGFs (3000–10,000) were plated in each well of a 96-well tissue culture plate with 100 μL of growth medium. The cells reached 40–50% confluence 24 h after plating. The medium was then replaced with 100 μL of fresh medium containing different concentrations of cyclosporine A (10, 10^2, 10^3, or 10^4 ng/mL) or with medium containing the solvent DMSO (control), and the cells were grown for 24 h. At the end of the incubation, 20 μL of MTS solution was added to each well, the cells were incubated at 37°C for 1–2 h and the absorbance was read at 490 nm (25).

Western blotting

Homogenates of primary cultured HGFs, lysed in lysis buffer, were centrifuged at 13,000 *g* for 15 min at 4°C, and boiled at 100°C for 10 min. The protein concentrations were determined with a protein microassay using the BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL, USA), and 40 μg protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 15% polyacrylamide gels with Tris/Tricine buffer and then electroblotted onto poly(vinylidene difluoride) membrane. Nonspecific binding was blocked by incubating the blots for 1 h in 5% bovine serum albumin. After six washes in phosphate-buffered saline containing 0.005% Tween 20, PCNA, CDK4, cyclin D1, Rb1, pRb1 and the internal control, α-tubulin, were detected by incubation of the membrane, overnight, at 4°C, with the appropriate primary antibody (26,27) and then for 1 h with the secondary horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, diluted 1:5000. The antibody-reactive proteins were detected using enhanced chemiluminescence. The expression of Rb1 and of pRb1 in the cyclosporine A-treated cells was calculated, using densitometric analysis, as a proportional increase or decrease relative to the levels observed in the control cells.

Statistical analysis

The Student's *t*-test was used to evaluate the differences between the control group and the cyclosporine A group in the expression of the mRNAs (relative densities) for *Pcna* and *Ccnd1*, obtained using real-time RT–PCR, and in the percentages of cells that stained positive after IHC treatment. One-way analysis of variance was used to evaluate the effect of cyclosporine A on cell proliferation in HGF cultures. A *p*-value of < 0.05 was selected as the level of significance.

Results

According to the results obtained using real-time PCR, the relative intensities of the *Pcna* and *Ccnd1* mRNAs were significantly stronger in the gingival specimens from cyclosporine A-treated animals than in those from the control rats (Fig. 1). The relative expression of the *Pcna* and *Ccnd1* mRNAs was 2.89-fold and 2.0-fold higher, respectively, in the cyclosporine A group than in the control group. The IHC-treated sections showed that significantly more gingival cells stained positively for cyclin D1 and pRb1 in the cyclosporine A-treated group than in the control group (Fig. 2). The mean number of CDK4-positive cells was higher in the cyclosporine A-treated group than in the control group, but the difference was not statistically significant.

In the *in vitro* MTS assay, the absorbance values were significantly higher for the cultures treated with 10^3 ng/mL of cyclosporine A, but

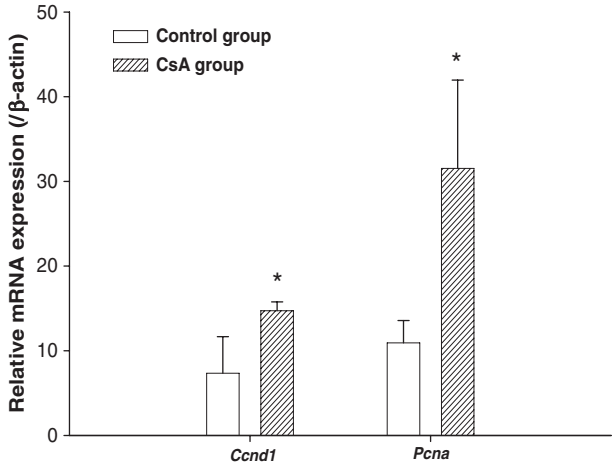

Fig. 1. Expression of *Ccnd1* and *Pcna* mRNAs, relative to that of *Actb* mRNA, in the gingivae of rats in the control and cyclosporine A groups (five rats in each group), assessed using real-time RT–PCR. Data are expressed as mean and SD; *significantly different from the control group at $p < 0.01$).

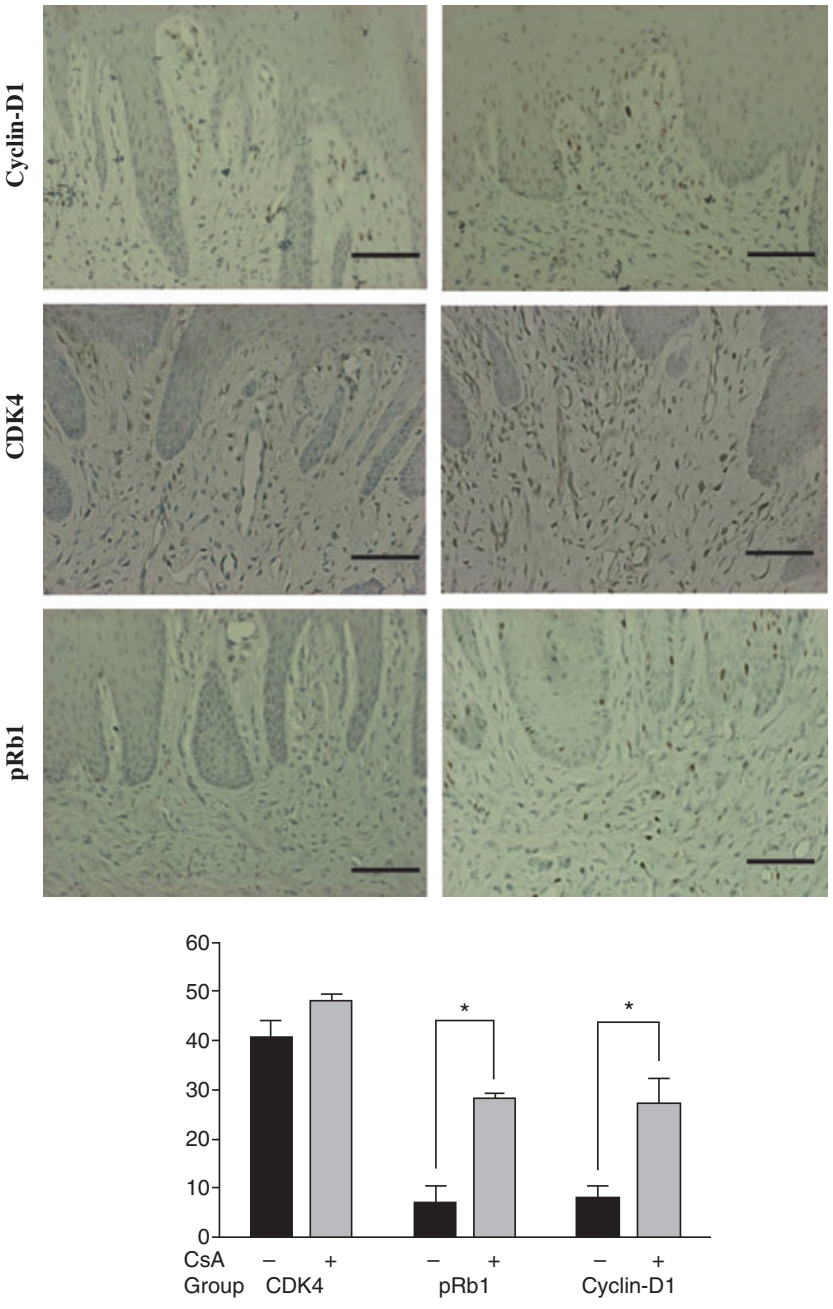

Fig. 2. The histomicrographs show cells staining positively for cyclin-dependent kinase 4 (CDK4), cyclin D1 and phosphorylated retinoblastoma protein 1 (pRb1) in the gingivae of the control (left column) and cyclosporine A-treated animals (right column; the scale bar represents 50 μm). The graph shows a comparison of the positively stained cells in the control and cyclosporine A-treated animal groups. Data are expressed as mean and SD, $n = 5$ rats per group. $*p < 0.05$.

significantly lower for the cultures treated with 10^4 ng/mL of cyclosporine A relative to the value for the DMSO-treated control (Fig. 3). A dose-dependent increase in PCNA protein expression was observed by western blotting analysis after the cyclosporine A treatments, and the expression of CDK4 and cyclin D1 was increased after treatment with cyclosporine A in the dose range of 10–10^3 ng/mL (Fig. 4). Moreover, the phosphorylation of Rb1 was enhanced after treatment with cyclosporine A at 10^2–10^3 ng/mL, but the levels of total Rb1 protein were similar after treatment with all doses of cyclosporine A (Fig. 4).

Discussion

The effects of cyclosporine A on parameters related to the cell cycle and on the levels of proteins involved in the control and progression of the cell cycle were recently evaluated in hepatocyte cultures (28). An increase in the percentage of cells involved in the S phase of the cell cycle, which correlated with increases in the levels of cyclins D1 and E, and of PCNA, but no modification in the expression of p27 (an inhibitory protein of CDKs), was observed in cultured hepatocytes after exposure to cyclosporine A. In the present study, not only CDK4, PCNA and cyclin D1, but also Rb1 and its phosphorylation (at Ser780 and Ser807/811) product pRB1, which promotes the G_1/S transition in the gingiva, were evaluated *in vivo* and *in vitro* after treatment with cyclosporine A. Our *in vivo* data demonstrated that the expression of cyclin D1, CDK4 and pRb1 proteins was higher in the cyclosporine A group than in the control group (Fig. 2), whereas our *in vitro* results showed increased expression of CDK4 and cyclin D1 and enhanced phosphorylation of Rb1 after treatment with cyclosporine A (Fig. 4). Moreover, significantly more HGFs were observed in the cultures treated with 10^3 ng/mL of cyclosporine A than in the cultures treated with DMSO (Fig. 3). However, in this study, certain results were still inconsistent. For instance, the expression of CDK4, cyclin D1 and pRb1 proteins was higher in HGFs treated with 10^2 ng/mL of cyclosporine A, but the expression of PCNA was greatest at a cyclosporine A concentration of 10^4 ng/mL. This may be attributable to the method used in the study to examine protein expression. For instance, CDK4, cyclin D1 and Rb1 regulate cell proliferation in the early stage of the cell cycle, but PCNA is present only in the late stage of cell proliferation, whereas the MTS assay measures the number of viable cell after cell proliferation is complete.

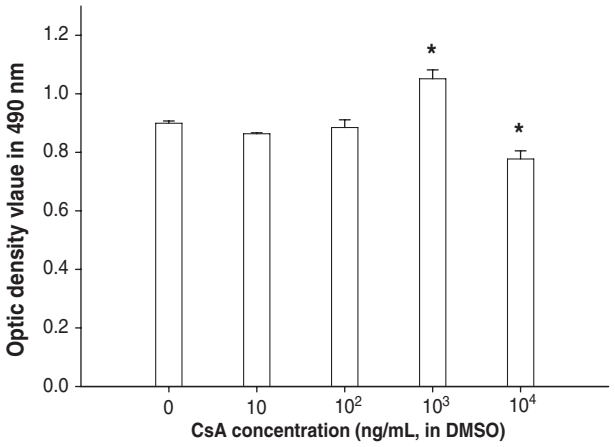

Fig. 3. Effects of cyclosporine A on the proliferation of human gingival fibroblasts (HGFs) cultures. Four concentrations of cyclosporine A (10, 10^2, 10^3 and 10^4 ng/mL) and the dimethylsulfoxide (DMSO; solvent) control were tested. Cell proliferation was measured using the MTS assay and the experiments were repeated three times. Data are expressed as mean and standard error; *significantly different from the DMSO control at $p < 0.05$ (Student's *t*-test).

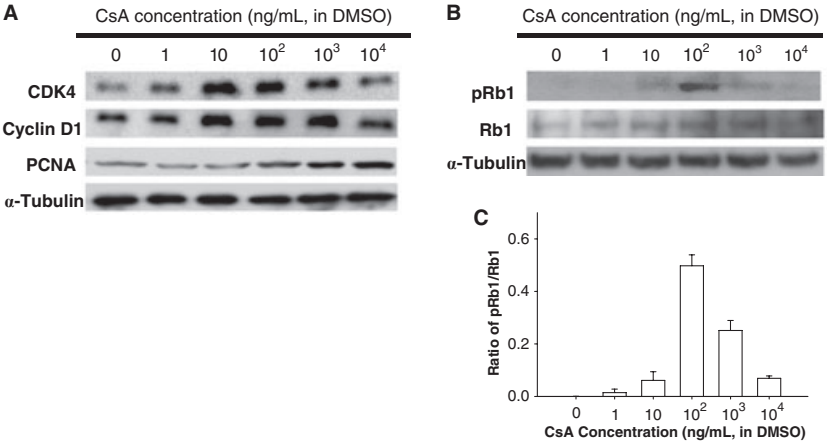

Fig. 4. Effects of cyclosporine A on the levels of the proliferation-associated proteins cyclin-dependent kinase 4 (CDK4), cyclin D1 and proliferating cell nuclear antigen (PCNA) (A) and on the retinoblastoma proteins retinoblastoma protein 1 (Rb1) and phosphorylated retinoblastoma protein 1 (pRb1) (B and C) in human gingival fibroblasts (HGFs). The HGFs were collected after treatment with dimethylsulfoxide (DMSO; solvent control) or cyclosporine A for 24 h, and expression of the proteins was examined by western blotting. The pRb1 : Rb1 expression ratios are summarized in (C) after normalization to α-tubulin (means and SD). The experiments were repeated three times.

Furthermore, the increased expression of PCNA after treatment with 10^4 ng/mL of cyclosporine A may be partly attributable to the accumulation of total protein present. Further detailed investigations are necessary to explain these phenomena.

In conclusion, the up-regulation of Rb1 phosphorylation in the gingiva during treatment with cyclosporine A was demonstrated *in vivo* and *in vitro*. Because the expression of CDK4, cyclin D1 and PCNA was also enhanced, we suggest that cyclosporine A, at the appropriate concentration, can cause gingival cells to enter the G_1/S phase transition and thereafter to proceed to the DNA-synthesis phase, leading to cell proliferation. These results support the growth-promoting effects of cyclosporine A in gingival cells.

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