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Growth factors and proliferation of cultured rat gingival cells in response to cyclosporin A

Takumasa Yoshida[1], Junji Nagata[2], Akira Yamane[2]
Departments of [1]Periodontics and Endodontics and [2]Pharmacology, Tsurumi University School of Dental Medicine, Yokohama, Japan

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Objective: The prominent side-effect of cyclosporin A, an immunosuppressive drug, in oral tissues is gingival outgrowth, although the exact mechanism underlying this side-effect is unclear. The main purposes of the present study were to determine whether cyclosporin A induced the gingival outgrowth by promoting proliferation of gingival cells and whether growth factors such as transforming growth factor-βs (TGF-βs), fibroblast growth factor-2 (FGF-2), platelet-derived growth factors (PDGFs), and insulin-like growth factors (IGFs) are involved in the possible changes in the proliferation of gingival cells induced by cyclosporin A.

Methods: Cells isolated from rat gingival tissues were cultured with cyclosporin A or IGF-I for 3 days. The effects of cyclosporin A or IGF-I on the proliferation of cultured rat gingival cells were analyzed with a CellTiter 96 proliferation assay kit. The mRNA expression levels for TGF-βs, FGF-2, PDGFs, IGFs, insulin-like growth factor receptors (IGFRs), and insulin-like growth factor binding proteins (IGFBPs) in the rat gingival cells treated with cyclosporin A were measured using competitive reverse transcription–polymerase chain reaction (RT–PCR).

Results: Cyclosporin A induced 23–25% ($p < 0.001$) increases in the proliferation of rat gingival cells and approximately 130% ($p < 0.05$) and 60% ($p < 0.05$) elevations in the mRNA expression levels for TGF-β1 and FGF-2, respectively. On the other hand, exogenous IGF-I induced 8–11% ($p < 0.05$) increases in the proliferation, but cyclosporin A induced 30–80% ($p < 0.05$–0.01) reductions in the mRNA expression levels for endogenous IGF-I, IGFR1, IGFBP2, IGFBP3, IGFBP5, and IGFBP6.

Conclusions: Cyclosporin A stimulates the proliferation of rat gingival cells. TGF-β1 and FGF-2 could be involved, but IGFs, IGFRs and IGFBPs could not be directly involved in this cyclosporin A induced-stimulation of the gingival cell proliferation.

Akira Yamane, Department of Pharmacology, Tsurumi University School of Dental Medicine, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama, 230-8501 Japan
Tel: +81 45 580 8452
Fax: +81 45 573 9599
e-mail: yamane-a@tsurumi-u.ac.jp

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Cyclosporin A is a cyclic polypeptide used as an immunosuppressive drug. Because cyclosporin A is especially effective in suppressing the cell-mediated immunoresponse, it is widely used to prevent organ transplant rejection and to treat various immunological diseases. The prominent side-effect of cyclosporin A therapy in oral tissues is gingival outgrowth (1, 2). Although there are a number of histological and biochemical studies focused on cy-

closporin A-induced gingival outgrowth, the precise mechanism underlying the pathogenesis of cyclosporin A-induced gingival outgrowth is still unclear. One hypothesis for this mechanism is that cyclosporin A induces gingival outgrowth by promoting proliferation of gingival cells. However, this hypothesis seems to be controversial; several studies have demonstrated that cyclosporin A stimulates the proliferation of human gingival fibroblasts (1, 3–5), whereas one study has reported that cyclosporin A suppresses this proliferation (2).

It is known that the expression levels for peptide growth factors such as transforming growth factor-β1 (TGF-β1), fibroblast growth factor-2 (FGF-2), and platelet derived growth factor-B (PDGF-B) are elevated in the drug-induced outgrowth of gingival tissues (6–9). Since these growth factors are reportedly potent mitogens in various kinds of tissues (10–12), it is implied that the elevations in the expression levels for these growth factors are involved in cyclosporin A-induced changes in the proliferation of gingival tissues.

Insulin-like growth factor (IGF)-I and -II are well known to play essential roles in significant biological activities such as proliferation, differentiation, apoptosis, and adaptation in various kinds of tissues (13). The actions of IGFs are mainly mediated via IGF receptor 1 (IGFR1) and are modulated by six binding proteins (IGFBPs), designated as IGFBP1 to IGFBP6 (13, 14). IGFR2 principally takes part in the turnover of IGF-II protein in tissues (15, 16). Despite many studies on the function of IGFs in various kinds of tissues, to our knowledge, there are no published data focused on the function of IGFs in cyclosporin A-induced gingival outgrowth.

The main purposes of the present study were to determine whether cyclosporin A induced the gingival outgrowth by promoting proliferation of gingival cells and whether growth factors such as TGF-βs, FGF-2, PDGFs, and IGFs are involved in the possible changes in the proliferation of gingival cells induced by cyclosporin A. We examined the effect of cyclosporin A on the proliferation of primary cultured cells obtained from normal rat gingival tissues. Then, we analyzed the mRNA expressions for TGF-βs, FGF-2, PDGFs, and IGFs in these cells. Furthermore, to determine whether IGFs, IGFRs and IGFBPs were implicated in the possible changes in the proliferation of gingival cells induced by cyclosporin A, we examined the mRNA expressions for IGFRs and IGFBPs in the primary cultured gingival cells and the effects of exogenous IGF-I on the proliferation of primary cultured gingival cells.

Material and methods

Cell culture

Buccal gingival tissues from three male Wistar rats at 8 weeks of age were minced and transferred to culture tissues. The tissues were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich Fine Chemicals, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, BD Biosciences Clontech, Palo Alto, CA, USA), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The outgrowing cells were subcultured and maintained in the same culture medium. The cells were used for experiments at 5–9 passages. These gingival cells used seem to be a mixture of several kinds of cell populations. The cells were seeded in Dulbecco's modified Eagle's medium containing 5 or 10% fetal bovine serum into 96-well, 24-well plates, or 3.5-cm dishes, and were allowed to attach to the wells or dishes for 1 day. The culture media were replaced with the media containing 0, 200, 400, or 800 ng/ml of cyclosporin A, or 0, 25, 50, or 100 ng/ml of IGF-I. The cultures were maintained for 3 days with changes of media every day.

Measurements of cell size and number

The effect of cyclosporin A (Sigma-Aldrich Fine Chemicals) or IGF-I (Life Technologies) on proliferation of the rat gingival cells was analyzed with a CellTiter 96 proliferation assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cells were seeded into 96-well plates (5×10^3 cells per well). Ten wells were used for each concentration of each drug ($n = 10$ in each concentration). The CellTiter 96 Aqueous One Solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) and phenazine ethosulfate] was added to the wells, and the MTS was reduced into a colored formazan product by the living cells. The quantity of formazan product was measured 3 h later at a wavelength of 490 nm, as the optical density at 490 nm is known to be directly proportional to the number of living cells (17). The results were confirmed by three independent experiments using cell populations obtained from three different rats.

To evaluate the effects of cyclosporin A or IGF-I on the size of the rat gingival cells, we measured the diameters of the cells using an automated cell analyzer (Vi-CELL™ Analyzer, Beckman Coulter, Inc., Fullerton, CA, USA). The cells were seeded into 24-well plates (7×10^3 cells per well). Six wells were used for each concentration of each drug ($n = 6$ in each concentration). The cells were rinsed with phosphate-buffered saline and incubated with 0. 25% trypsin and 0.02% EDTA. The diameters of the released cells were assayed using the automated cell analyzer. The areas of the released cells were measured by image analysis using the automated cell analyzer, and the diameters were calculated based on the assumption that the cells were spherical in shape. The results were confirmed by two independent experiments using cells obtained from two different rats.

RNA extraction, reverse transcription (RT), and competitive polymerase chain reaction (PCR) amplification

The cells were seeded in Dulbecco's modified Eagle's medium containing

10% fetal bovine serum into 3.5-cm dishes (2×10^4 cells per dish). Three dishes were used for each concentration of cyclosporin A ($n = 3$ in each concentration). After the culture, the cells were mechanically removed from the dishes. Total RNA extraction, RT, and competitive PCR amplification were performed as previously described (18). The total RNA extraction was performed according to the manufacturer's specifications (Trizol, Life Technologies). The RNA was treated with 2 U of ribonuclease-free deoxyribonuclease I (Life Technologies), and was then reverse transcribed with 200 U of reverse transcriptase (SuperScript II, Life Technologies).

In the conventional PCR technique, a small difference in the starting amount of target DNA can result in a large change in the yield of the final product due to the exponential nature of the PCR reaction. A plateau effect after many cycles can lead to an inaccurate estimation of final product yield. Furthermore, since the PCR amplification depends on the reaction efficiency, small changes in the efficiency can lead to major differences in the final product yield. To overcome these problems, the competitor (internal standard), which has the same primer sequences as those of the target DNA at the 5′ and 3′ ends, was amplified simultaneously with the target (18–21). The competitors were constructed according to the manufacturer's instructions in the Competitive DNA Construction Kit (TaKaRa Biochemicals, Shiga, Japan) and were amplified with 50 ng of the total cDNA in the presence of a primer pair specific to the target genes in a thermal cycler (TP3000, TaKaRa Biochemicals). Table 1 shows the primer sequences and product sizes for TGF-βs, FGF-2, and PDGFs; those for IGFs, IGFRs, IGFBPs, and S16 were previously reported (22–24). The amplification products were separated by electrophoresis on an agarose gel containing ethidium bromide. The fluorescent intensities of the bands of the target cDNAs and their respective competitors were measured by an image analyzer (Molecular Imager FX, Bio-Rad, Hercules, CA, USA). We then calculated the ratios of the fluorescent intensities of the target cDNA bands to those of their respective competitors. The logarithmic value of the fluorescent intensity ratio was used to calculate the amount of endogenous gene mRNA based on the line formula derived from a standard curve for each target gene. The standard curve was generated as described previously (18, 24). The quantity of each target mRNA was normalized by the quantity of S16 (ribosomal protein). The resulting ratio value was expressed as a percentage value relative to the mean value of each target gene at 0 ng/ml of cyclosporin A. The results were confirmed by two independent experiments using cell populations obtained from two different rats.

Table 1. Sequences of target gene-specific polymerase chain reaction primers, target and competitor sizes

Target genes	Sequences	References
TGF-β1		
Forward	5′-CTAATGGTGGACCGCAACAAC-3′	(32)
Reverse	5′-CGGTTCATGTCATGGATGGTG-3′	
Target size	431 bp	
Competitor size	342 bp	
TGF-β2		
Forward	5′-AAAATGCCATCCCGCCCACTT-3′	(32)
Reverse	5′-CATCAATACCTGCAAATCTCG-3′	
Target size	420 bp	
Competitor size	342 bp	
TGF-β3		
Forward	5′-TGGCGGAGCACAATGAACTGG-3′	(32)
Reverse	5′-CCTTTGAATTTGATTTCCATC-3′	
Target size	415 bp	
Competitor size	342 bp	
FGF-2		
Forward	5′-CCACCAGGCCACTTCAAGG-3′	(33)
Reverse	5′-AACTCCTCTCTCTTCTGC-3′	
Target size	150 bp	
Competitor size	237 bp	
PDGF-A		
Forward	5′-TCAAGGTGGCCAAAGTGGAG-3′	(34)
Reverse	5′-CTCTCTGTGACAAGGAAGCT-3′	
Target size	370 bp	
Competitor size	290 bp	
PDGF-B		
Forward	5′-ATCGCCGAGTGCAAGACGCG-3′	(34)
Reverse	5′-AAGCACCATTGGCCGTCCGA-3′	
Target size	582 bp	
Competitor size	440 bp	

TGF, transforming growth factor; FGF-2, fibroblast growth factor-2; PDGF, platelet-derived growth factor.

Statistical analysis

For multiple comparisons, Dunnett's method was used to compare the mean values between two groups.

Results

Effects of cyclosporin A on the size and proliferation of rat gingival cells

Figure 1A shows the rat gingival cells cultured for 3 days in 10% fetal bovine serum–Dulbecco's modified Eagle's medium containing 0 or 800 ng/ml of cyclosporin A. No marked difference in the shape or size was observed between the control cells and 800 ng/ml of cyclosporin A-treated cells. To evaluate the effects of cyclosporin A on the size of the rat gingival cells, we measured the cell diameters (Fig. 1B) based on

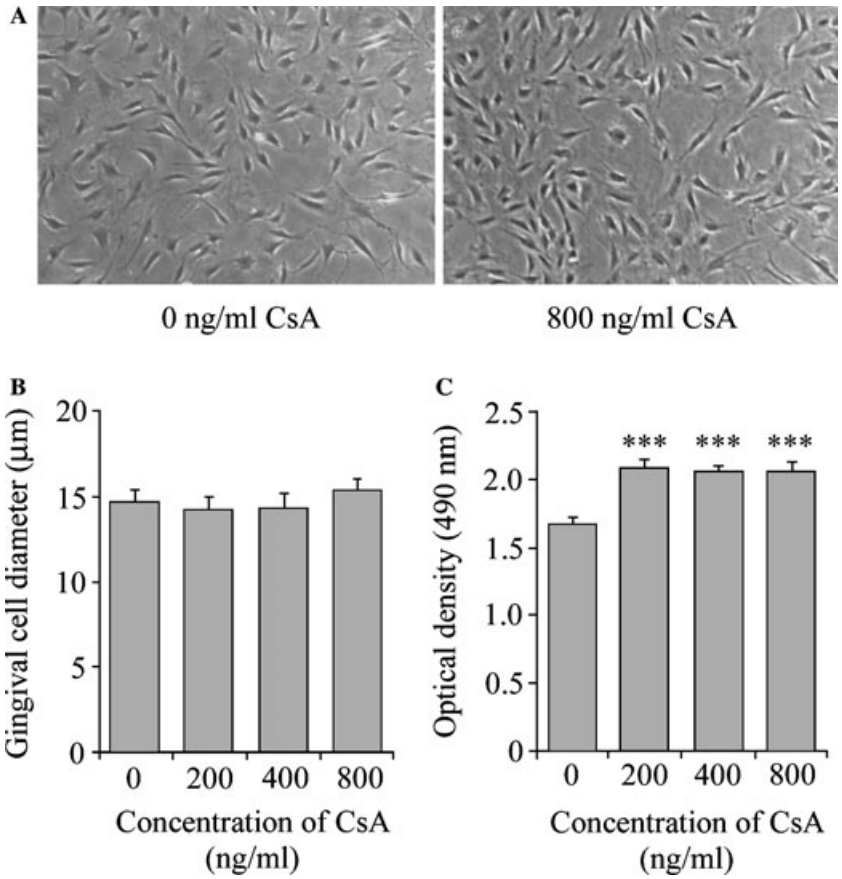

Fig. 1. Effects of cyclosporin A (CsA) on the shape, size, and proliferation of cultured rat gingival cells. Rat gingival cells cultured for 3 days in 10% fetal bovine serum–Dulbecco's modified Eagle's medium containing 0 or 800 ng/ml of CsA (A). The diameters of the rat gingival cells (B) and the optical density at 490 nm for formazan produced by the gingival cells (C) cultured in 10% fetal bovine serum–Dulbecco's modified Eagle's medium containing 0, 200, 400, or 800 ng/ml CsA. Each column and its vertical bar represent the mean + 1 SD of 6 (B) and 10 (C) wells. The results were confirmed by two (B) or three (C) independent experiments using cell populations obtained from two or three different rats. Statistically significant difference from the mean value at 0 ng/ml of CsA; ***$p < 0.001$.

the area of the released cells assuming a spherical cell shape. The treatments with 200–800 ng/ml of cyclosporin A did not affect the diameter of the rat gingival cells. The effects of cyclosporin A on the proliferation of the gingival cells were assessed by measuring the quantity of a formazan product, known to be reduced by living cells, at a wavelength of 490 nm (Fig. 1C). The treatments with 200–800 ng/ml of cyclosporin A induced 23–25% ($p < 0.001$) increases in the optical density at 490 nm for the formazan product. These results suggest that cyclosporin A stimulates the proliferation of gingival cells, but does not affect their size.

Effects of cyclosporin A on the mRNA expressions for TGF-βs, FGF-2, PDGFS, and IGFS in the rat gingival cells

To investigate the involvement of TGF-βs (Fig. 2), FGF-2, PDGFs (Fig. 3), and IGFs (Fig. 4) in the cyclosporin A-induced stimulation of rat gingival cell proliferation, the mRNA levels for these growth factors were measured in the rat gingival cells cultured for 3 days in 10% fetal bovine serum–Dulbecco's modified Eagle's medium containing 0–800 ng/ml of cyclosporin A by competitive RT–PCR. Figure 2A shows a typical example of a gel electrophoretic pattern for TGF-β1 competitive PCR products. As the concentration of cyclosporin A increased, the ratios of the fluorescent intensities of the TGF-β1 bands (upper bands) to those of their respective competitor bands (lower bands) appeared to be greater. Image analyses of the PCR bands indicated that the treatments with 400 and 800 ng/ml of cyclosporin A induced 80% ($p < 0.05$) and 129% ($p < 0.05$) increases in the mRNA expression levels for TGF-β1, respectively (Fig. 2B), whereas the treatments with cyclosporin A did not induce a significant change in the mRNA expression levels for TGF-β3 (Fig. 2C). There was no TGF-β2 detected, probably due to the small amount of mRNA expression. The treatments with 400 and 800 ng/ml of cyclosporin A induced approximately 60% ($p < 0.05$) increases in the mRNA expression levels for FGF-2 (Fig. 3A). The treatments with cyclosporin A did not induce a significant change in the mRNA expression levels for PDGF-A (Fig. 3B) and no PDGF-B mRNA was detected. The treatments with 800 ng/ml of cyclosporin A induced a 33% ($p < 0.05$) decrease in the mRNA expression levels for IGF-I (Fig. 4A), whereas the treatments with cyclosporin A did not significantly change the mRNA expression level for IGF-II (Fig. 4B).

Effects of cyclosporin A on the mRNA expressions for IGFRs and IGFBPs

As a cyclosporin A-induced decrease in the mRNA expression level for IGF-I was found in association with the stimulatory effect of cyclosporin A on the proliferation of rat gingival cells in the present study, we analyzed the mRNA expression levels for IGFRs and IGFBPs, which mediate and modulate the actions of IGFs (Figs 5 and 6). The mRNA expression levels for IGFR1 at 200–800 ng/ml of cyclosporin A were approximately 60% less than that at 0 ng/ml ($p < 0.05$) (Fig. 5A). The mean values of the mRNA expression levels for IGFR2 at 200–800 ng/ml of cyclosporin A were

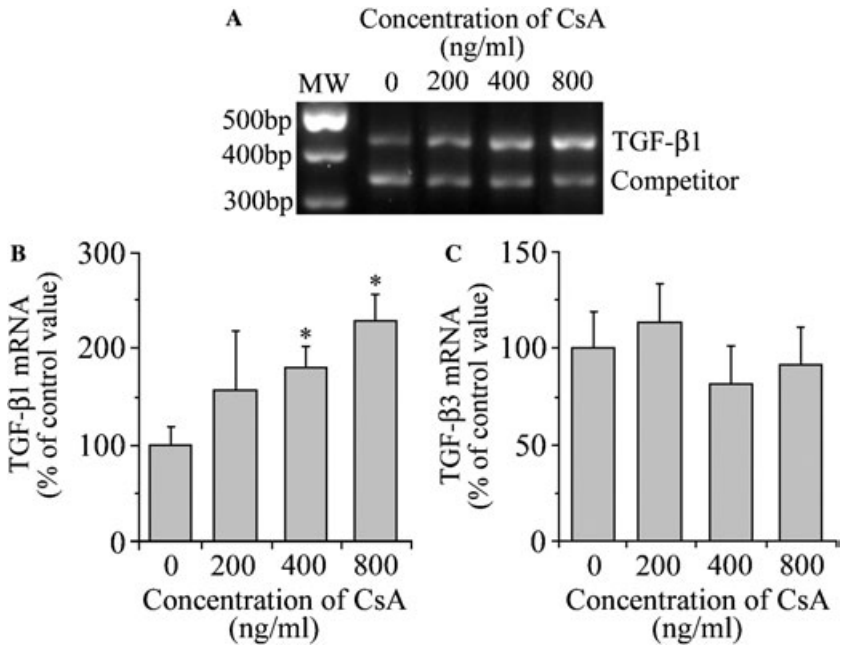

Fig. 2. Effects of cyclosporin A (CsA) on the mRNA expression levels for transforming growth factor-βs (TGF-βs) in the rat gingival cells. Typical example of a gel electrophoretic pattern for TGF-β1 competitive polymerase chain reaction (PCR) products of rat gingival cells cultured in 10% fetal bovine serum–Dulbecco's modified Eagle's medium containing 0, 200, 400, or 800 ng/ml of CsA (A). Relative changes in the mRNA expression levels for TGF-β1 (B) and TGF-β3 (C) assessed by using competitive reverse transcription–PCR. No TGF-β2 mRNA was detected. Each column and its vertical bar represent the mean + 1 SD of three wells. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml. The results were confirmed by two independent experiments using cells obtained from two different rats. Statistically significant difference from the mean value at 0 ng/ml of CsA; $*p < 0.05$. MW, molecular weight.

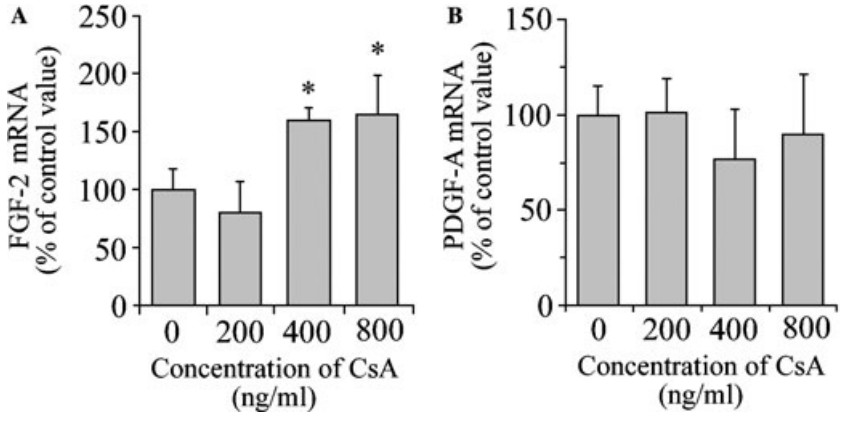

Fig. 3. Effects of of cyclosporin A (CsA) on the mRNA expression levels for fibroblast growth factor-2 (FGF-2) and platelet-derived growth factors (PDGFs) in the rat gingival cells. Relative changes in the mRNA expression levels for FGF-2 (A) and PDGF-A (B) assessed by using competitive reverse transcription-polymerase chain reaction. No PDGF-B mRNA was detected. Each column and its vertical bar represent the mean + 1 SD of three wells. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml. The results were confirmed by two independent experiments using cell populations obtained from two different rats. Statistically significant difference from the mean value at 0 ng/ml of CsA; $*p < 0.05$.

also less than that at 0 ng/ml, although these decreases were not statistically significant (Fig. 5B). The mRNA expression levels for IGFBP2 and IGFBP3 at 200–800 ng/ml of cyclosporin A were approximately 50–80% ($p < 0.05$–0.01) less than that at 0 ng/ml (Figs 6A and B). None of the concentrations of cyclosporin A significantly changed the mRNA expression levels for IGFBP4 (Fig. 6C). The treatments with 400 and 800 ng/ml of cyclosporin A induced 65% ($p < 0.05$) and 78% ($p < 0.01$) decreases in the mRNA expression levels for IGFBP5, respectively (Fig. 6D). Treatments with 200–800 ng/ml of cyclosporin A induced decreases in the mRNA expression level for IGFBP6 in a dose-dependent manner; only the 53% decrease at 800 ng/ml was statistically significant ($p < 0.05$, Fig. 6E). No IGFBP1 mRNA was detected due to the small amount of mRNA.

Effects of IGF-I on the proliferation and size of rat gingival cells

Decreases in the mRNA expression levels for IGF-I, IGFR1, IGFBP2, IGFBP3, IGFBP5, and IGFBP6 were observed in the rat gingival cells treated with cyclosporin A. Thus, to further understand the involvement of IGF in the cyclosporin A-induced gingival outgrowth, we analyzed the proliferation of rat gingival cells cultured in 10% (Fig. 7A) or 5% (Fig. 7B) fetal bovine serum–Dulbecco's modified Eagle's medium containing 0–100 ng/ml of IGF-I by measuring the quantity of a formazan product, known to be reduced by living cells, at a wavelength of 490 nm. In the rat gingival cells cultured in 10% fetal bovine serum–Dulbecco's modified Eagle's medium, the treatment with only 100 ng/ml of IGF-I induced an 8% increase in the optical density for the formazan product ($p < 0.05$) (Fig. 7A). In the rat gingival cells cultured in 5% fetal bovine serum–Dulbecco's modified Eagle's medium, the optical densities at 490 nm at 25–100 ng/ml of IGF-I were 10–11% greater than that at 0 ng/ml ($p < 0.05$). None of the

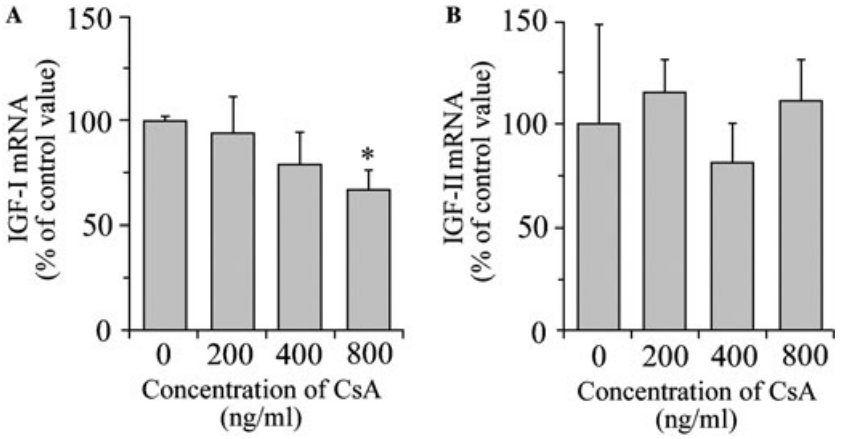

Fig. 4. Effects of of cyclosporin A (CsA) on the mRNA expression levels for insulin-like growth factors (IGFs) in the rat gingival cells. Relative changes in the mRNA expression levels for IGF-I (A) and IGF-II (B) assessed by using competitive reverse transcription-polymerase chain reaction. Each column and its vertical bar represent the mean + 1 SD of three wells. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml. The results were confirmed by two independent experiments using cell populations obtained from two different rats. Statistically significant difference from the mean value at 0 ng/ml of CsA; $*p < 0.05$.

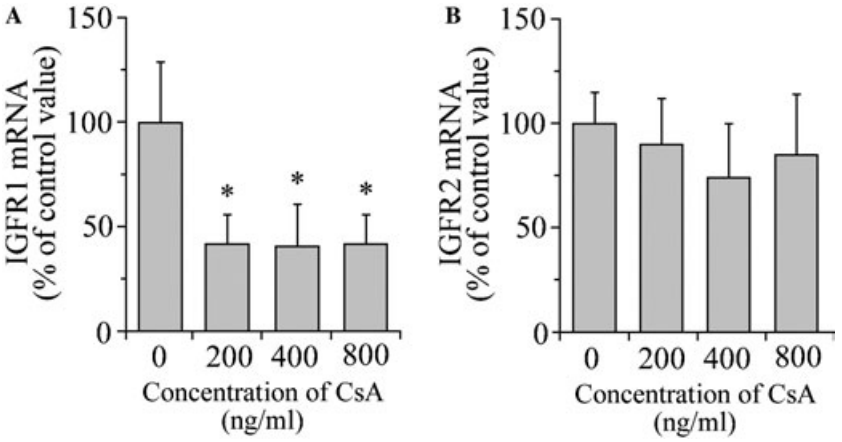

Fig. 5. Effects of of cyclosporin A (CsA) on the mRNA expression levels for insulin-like growth factor receptors (IGFRs) in the rat gingival cells. Relative changes in the mRNA expression levels for IGFR1 (A) and IGFR2 (B) assessed by using competitive reverse transcription-polymerase chain reaction. Each column and its vertical bar represent the mean + 1 SD of three wells. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml. The results were confirmed by two independent experiments using cell populations obtained from two different rats. Statistically significant difference from the mean value at 0 ng/ml of CsA; $*p < 0.05$.

concentrations of cyclosporin A significantly changed the diameters of cells cultured in both 5% and 10% fetal bovine serum–Dulbecco's modified Eagle's medium (Figs 7C and D). These results suggest that IGF-I slightly stimulated the proliferation of rat gingival cells, but did not affect their size.

Discussion

The effects of the cyclosporin A on the proliferation of human gingival fibroblasts are controversial; several studies have demonstrated that cyclosporin A stimulates the proliferation (1, 3–5), whereas one study has reported that cyclosporin A suppresses this proliferation (2). In these studies, gingival cells obtained from male or female humans with a wide range of ages from 17 to 67 years old were used. In addition, it seems very difficult to collect human gingival tissues with clinically same conditions. These varieties could cause the discrepancy among these studies. Since it is easy to obtain gingival cells with the same age and sex, and clinically similar conditions, rat gingival cells were used in the present study.

We observed 23–25% increases in the proliferation of rat gingival cells (Fig. 1C), suggesting that the enhancement of gingival proliferation by cyclosporin A contributes to the cyclosporin A-induced gingival outgrowth. However, the 23–25% increases seem to be small in comparison with the report that the administration of cyclosporin A to rats enlarged the cross-sectional area of the gingival tissue by more than 150% (25). In addition, an abnormal synthesis of collagen and other extracellular matrix components (3, 4, 26), and a decline of collagen turnover via changes in the matrix metalloproteinases and tissue inhibitors of metalloproteinase (9, 27, 28) reportedly occur in association with cyclosporin A-induced gingival outgrowth. Thus, the mechanism by which cyclosporin A induces the gingival outgrowth is probably comprised of several factors, and includes the enhancement of gingival cell proliferation.

We observed an increase in mRNA expression levels for TGF-β1 (Fig. 2B) and FGF-2 (Fig. 3A) in association with the stimulatory effect of cyclosporin A on the proliferation of rat gingival cells (Fig. 1C), suggesting that cyclosporin A stimulates the proliferation by changing the expression levels for TGF-β1 and FGF-2. There is supporting evidence that exogenous TGF-β1 and FGF-2 stimulate the proliferation of cultured human gingival fibroblasts (5, 29). In addition, it has been reported that exogenous FGF-2 modulates the collagen metabolism of human gingival fibroblasts (30) and that the inhibition of TGF-β1 by antisense oligonucleotide increases the expression of collagen degrading

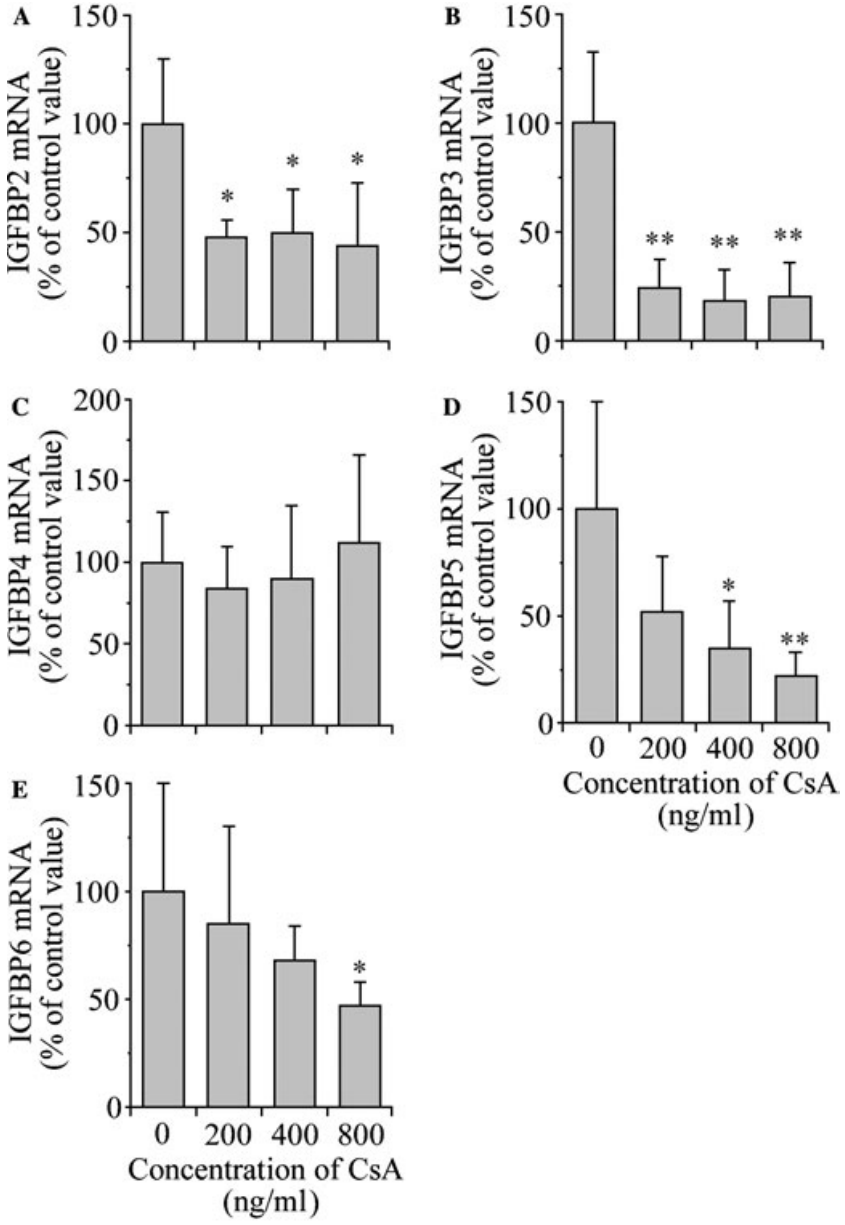

Fig. 6. Effects of of cyclosporin A (CsA) on the mRNA expression levels for insulin-like growth factor binding proteins (IGFBPs) in the rat gingival cells. Relative changes in the mRNA expression levels for IGFBP2 (A), IGFBP3 (B), IGFBP4 (C), IGFBP5 (D), and IGFBP6 (E) assessed by using competitive reverse transcription-polymerase chain reaction. Each column and its vertical bar represent the mean + 1 SD of three wells. The vertical axis is expressed as a percentage of the mean value at 0 ng/ml. The results were confirmed by two independent experiments using cell populations obtained from two different rats. Statistically significant differences from the mean value at 0 ng/ml of CsA; $*p < 0.05$, $**p < 0.01$.

enzymes in human gingival fibroblasts (9). Thus, cyclosporin A may alter the metabolism of collagen and the expressions of collagen degrading enzymes in rat gingival cells by elevating the expressions of FGF-2 and TGF-β1.

It has been reported that cyclosporin A stimulates macrophages to produce PDGF-B in human gingival tissues (7, 8). In the present study, no PDGF-B mRNA was detected, probably due to the very small amount of its mRNA expressed in the rat gingival cells. Our findings were confirmed by three independent experiments using gingival cells obtained from three different rats, and they suggested that our cultured gingival cell populations did not contain a macrophage population producing PDGF-B and did not produce PDGF-B themselves.

Although IGFs, IGFRs and IGFBPs (i.e. IGF system) are well known to play essential roles in significant biological activities such as proliferation, differentiation, apoptosis, and adaptation in various kinds of tissues (13), there is no published data focused on their functions in the cyclosporin A-induced outgrowth of gingival tissues. Thus we examined the effects of cyclosporin A on the expressions of endogenous IGFs, IGFRs and IGFBPs in the rat gingival cells (Figs 4, 5 and 6) and the effect of exogenous IGF-I on the proliferation of the rat gingival cells (Fig. 7). Although exogenous IGF-I induced 8–11% increases in the proliferation of the rat gingival cells, cyclosporin A induced a significant and marked reduction in the mRNA expression level for most of IGF system except for IGF-II, IGFR2 and IGFBP4 in the cultured rat gingival cells. These results suggest that the IGF systems could not be directly involved in the stimulatory effect of cyclosporin A on the proliferation of gingival cells. Probably, the down-regulations of the IGF-system seem to occur because the IGF system is not directly needed for the stimulatory effect of cyclosporin A on the proliferation of gingival cells. In the present study, we observed a cyclosporin A-induced increase in the expression levels of TGF-β1 (Fig. 2B) and FGF-2 (Fig. 3A). In addition, cyclosporin A is reported to induce an increase in the expression of PDGF-B (7, 8) and interleukin-6 (31). Thus, a complicated cytokine network that includes TGF-β1, FGF-2, PDGF-B and interleukin-6 may be implicated in the cyclosporin A-induced gingival outgrowth.

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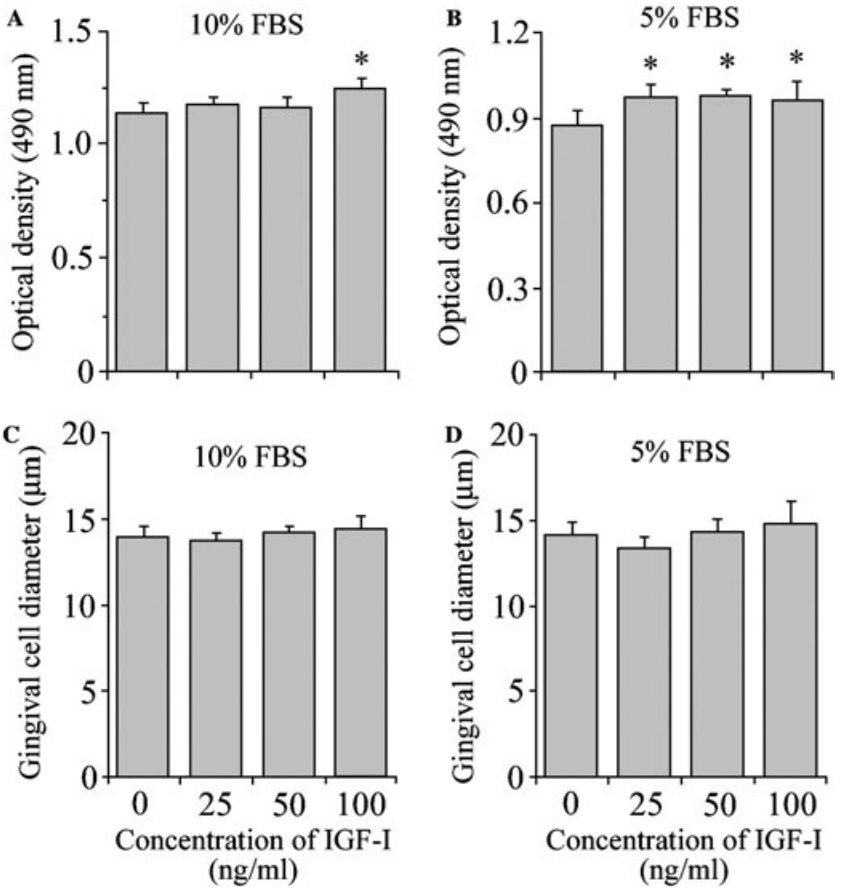

Fig. 7. Effects of insulin-like growth factor-I (IGF-I) on size and proliferation of cultured rat gingival cells. The optical density at 490 nm for a formazan product reduced by the gingival cells (A, B) and the diameters of rat gingival cells (C, D) cultured in 10% (A, C) or 5% (B, D) fetal bovine serum (FBS)–Dulbecco's modified Eagle's medium containing 0, 25, 50, or 100 ng/ml IGF-I. Each column and its vertical bar represent the mean + 1 SD of 10 (A, B) and 6 (C, D) wells. The results were confirmed by three (A, B) or two (C, D) independent experiments using cell populations obtained from three or two different rats. Statistically significant difference from the mean value at 0 ng/ml of IGF-I; $*p < 0.05$.

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