J Periodont Res 2004; 39; 320–326
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JOURNAL OF PERIODONTAL RESEARCH
doi: 10.1111/j.1600-0765.2004.00746.x

Long-term cyclosporin A exposure suppresses cathepsin-B and -L activity in gingival fibroblasts

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Yamaguchi M, Naruishi K, Yamada-Naruishi H, Omori K, Nishimura F, Takashiba S. Long-term cyclosporin A exposure suppresses cathepsin-B and -L activity in gingival fibroblasts. J Periodont Res 2004; 39; 320–326. © Blackwell Munksgaard 2004

Background: Gingival overgrowth is a common side-effect following administration of cyclosporin A. We reported previously that lysosomal protease cathepsin-L activity, but not cathepsin-B, was significantly suppressed by short-term cyclosporin A exposure in human gingival fibroblasts. Although this suppression may lead to decreased degradation of gingival connective tissue, a net increase in matrix proteins, and gingival overgrowth, the effects of cyclosporin A need to be more elucidated, considering the long-term use for patients following organ transplantation.

Objective: The aim of the present study was to evaluate the effects of clinically relevant doses of cyclosporin A on cultured human gingival fibroblasts. We evaluated the effects of long-term cyclosporin A exposure on cell proliferation, mRNA expression of various proteases and both cathepsin-B and -L activity in human gingival fibroblasts.

Materials and Methods: Human gingival fibroblasts were isolated from three donors' healthy gingiva and cultured from five to eight passages with or without 200 ng/ml of cyclosporin A. Proliferative activity of cyclosporin A-treated cells was examined using MTT assay. Total RNA and cellular proteins were collected for semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis and for measurement of the cathepsin-B and -L activity.

Results: Long-term cyclosporin A exposure had no effects on cell proliferation. Accumulation of cathepsin-B, -H and -L mRNA was markedly suppressed by long-term cyclosporin A exposure, whereas accumulation of another lysosomal enzyme *N*-acetyl-β-D-glucosaminidase mRNA, which is involved in remodeling of gingival epithelium, was not apparently impaired in cyclosporin A-treated cells. Accumulation of matrix metalloprotease-1 (MMP-1) and tissue inhibitor of matrix metalloprotease-1 (TIMP-1) mRNA, which are involved in remodeling of extracellular matrix, also was not impaired. In addition, we demonstrated that long-term cyclosporin A exposure significantly suppressed not only the activity of the active form of cathepsin-(B + L) compared to the activity in non-treated cells ($p = 0.0458$), but also the activity of the active form of cathepsin-B ($p < 0.0001$) in human gingival fibroblasts.

Conclusion: The decreased ability of protein degradation by not only cathepsin-L but also cathepsin-B is, at least, one of the several factors developing the cyclosporin A-induced gingival overgrowth.

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Key words: cathepsins; cyclosporin A; gingival fibroblasts; gingival overgrowth

Accepted for publication March 25, 2004

Cyclosporin A has been used topically as an immunosuppressive agent for its ability to reduce the cytokine expression from T cells and thus reduces local inflammation (1). There is growing evidence that cyclosporin A could affect the biological functions of some non-immune cell types, including endothelial cells and human gingival fibroblasts (2, 3). Gingival overgrowth is one of the common side-effects of the systemic use of cyclosporin A, probably caused by direct or indirect effects of the drug on human gingival fibroblasts, and the feature is an enlargement of the gingival connective tissue (4). Considering the improvement of medical science, cyclosporin A should be used frequently for patients following organ transplantation. Therefore it is assumed that there will be an increase in the number of patients developing gingival overgrowth. To make matters worse, enlarged gingival tissue may allow further accumulation of oral bacteria into the periodontal pockets, suggesting that this unwanted side-effect may affect the clinical course of marginal periodontitis and subsequent systemic health if complicated.

The imbalance between the production of newly synthesized matrix proteins and their inadequate removal or degradation results in excessive accumulation of matrix protein (1). Lysosomal enzymes cathepsin-B and -L are widely expressed as cysteine proteases implicated in both intracellular proteolysis and extracellular matrix remodeling (5). We previously reported that cyclosporin A suppressed the activity of cathepsin-L, but not cathepsin-B in cultured human gingival fibroblasts (6). These results showed that cathepsin-L plays an important role in the development of gingival overgrowth *in vitro*, although human gingival fibroblasts used in the study had been stimulated by short-term cyclosporin A exposure. Furthermore, we used cathepsin-L knockout mice as a model system to examine the *in vivo* relevance of these findings. As expected, the mice deficient in the cathepsin-L gene were characterized by thickened gingival tissues compared with wild-type control mice (7). These results suggest that cathepsin-L is a key molecule in the pathogenesis of cyclosporin A-induced gingival overgrowth.

Our previous findings should be useful for understanding the pathogenesis of gingival overgrowth. However, the biological effect of long-term cyclosporin A exposure on human gingival fibroblasts needs to be more elucidated, because long-term treatment with cyclosporin A is indispensable to patients following organ transplantation (8–10). The aim of the present study was to evaluate the direct effects of clinically relevant doses of cyclosporin A on human gingival fibroblast functions *in vitro* by assessing cell proliferation, mRNA expression of various proteases and both cathepsin-B and -L activity. We believe that this approach might have wide implications for underlying mechanisms of gingival overgrowth in general.

Materials and methods

Reagents

Cyclosporin A and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Z-Phe-Arg-MCA, Z-Arg-Arg-MCA (enzyme synthetic substrates) and CA-074 (cathepsin-B specific inhibitor) were obtained from the Peptide Institute (Osaka, Japan).

Cell culture

Human gingival fibroblasts were isolated from the non-inflamed gingival tissue obtained from three donors as described previously (11). Informed consent was obtained from each donor before participation. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM: Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY, USA) and 10 mg/ml of gentamycin (Life Technologies) with or without cyclosporin A between five and eight passages, while these cells were actively growing. Medium was changed daily and cyclosporin A was re-added to the culture to maintain a concentration of 200 ng/ml; the dose used was within the clinical dose range (12). cyclosporin A was diluted with dimethylsulfoxide and control culture contained the same concentration of dimethylsulfoxide throughout the experiments.

MTT cell proliferation assay

MTT assay was performed as described previously (13). In short, cyclosporin A-treated cells were seeded in each well of a 96-well plate (Corning Costar, Cambridge, MA, USA) in a final volume of 100 μl of DMEM medium supplemented with 10% fetal bovine serum. After cells reached subconfluence, the medium was changed to fetal bovine serum-free DMEM and kept for 24 h. At the end of the treatments, MTT (final concentration: 0.5 mg/ml) was added to each well and incubated for 4 h in a humidified atmosphere prior to the addition of 100 μl of acid solution (0.04 N HCl in isopropanol) into each well. The reaction mixture on each well of the 96-well culture plate was measured fluorometrically using the Auto plate reader (MTP-120: excitation at 570 nm, emission at 630 nm; Corona Electric, Co., Ltd. Hitachinaka, Japan). Each sample was tested in three individual wells.

Reverse transcription-polymerase chain reaction (RT-PCR)

Detection of the mRNA encoding matrix metalloprotease (MMP)-1, tissue inhibitor of matrix metalloprotease (TIMP)-1, cathepsin-B, -H, -L, *N*-acetyl-β-D-glucosaminidase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the cells was performed by semi-quantitative RT-PCR (6). Total RNA was isolated from cells using TRIZOL®LS Reagent (Life Technologies), and first-strand cDNA was synthesized with SuperScript™ Preamplification System (Life Technologies) using oligo $(dT)_{12-18}$ primer (Life Technologies) according to the manufacturer's instruction. PCR was performed using AmpliTaq Gold® DNA polymerase (Perkin Elmer Cetus, Emeryville, CA, USA) in 25 μl of reaction cocktail containing 1.5 mM of

$MgCl_2$ according to the manufacturer's instruction. The sets of primers used were as follows: MMP-1: 5′-CCCAA GGACATCTACAGCTC-3′ for forward and 5′-GCTCTCTGGGATCAA CGTCA-3′ for reverse; TIMP-1: 5′-CT GTTGTTGCTGTGGCTGAT-3′ for forward and 5′-TCCGTCCACAAGC AATGAGT-3′ for reverse; *N*-acetyl-β-D-glucosaminidase: 5′-ACTATGAGG AGGCAAGAAGC-3′ for forward and 5′-GCCCTGGAATTAGCGGAAAA-3′ for reverse; cathepsin B: 5′-GTTAC AGTGCAGACAGGCCA-3′ for forward and 5′-GTTTCCTTTTGAGC CGCGTC-3′ for reverse; cathepsin H: 5′-TGGCTGTTGGGTATGGAGAA-3′ for forward and 5′-AAGGACACT AAGGCACATGG-3′ for reverse; cathepsin L: 5′-AGTGTGGCTCTTG TTGGGCT-3′ for forward and 5′-GC CAACCACCAGCATAGCAT-3′ for reverse; GAPDH: 5′-TGGTATCGTG GAAGGACTCATGAC-3′ for forward and 5′-ATGCCAGTGAGCTT CCCGTTCAGC-3′ for reverse. PCR cycle characteristics were 25–30 cycles of denaturation (97°C, 1 min), annealing (59°C for GAPDH; 58°C for *N*-acetyl-β-D-glucosaminidase; 57°C for cathepsin-B, -H and TIMP-1; 56°C for cathepsin-L; 51°C for MMP-1; 1 min for each) and extension (72°C, 1 min). One-tenth of the cDNA sample was used for PCR in a thermal cycler (Program Temp Control System PC-700; ASTEC, Fukuoka, Japan). One-fifth of the amplified product was subjected to agarose gel electrophoresis and visualized by staining the gel with ethidium bromide (5 μg/ml). The amount of mRNA encoding MMP-1, TIMP-1, cathepsin-B, -H and -L, and *N*-acetyl-β-D-glucosaminidase was estimated by the intensity of the staining of amplified cDNA rerative to that encoding GAPDH with NIH Image software (version 1.62).

Cathepsin activity

The activities of the active forms of cathepsin-(B + L) and cathepsin-B in human gingival fibroblasts were determined fluorometrically using Z-Phe-Arg-MCA (for B + L assay) or Z-Arg-Arg-MCA (for B assay) as the substrates, whereas total activities including the latent form of cathepsin-(B + L) or that of cathepsin-B were determined following activation by limited proteolysis with pepsin at a final concentration of 50 μg/ml as described previously (6). Cells were cultured from five to eight passages with or without cyclosporin A and were washed twice with phosphate-buffered saline solution and scraped into 2 ml of ice-cold 50 mM sodium acetate buffer (pH 5.2) containing 1 mM EDTA and 0.1 M NaCl. Then the cell membranes were permeabilized by sonication. The activities of active forms of cathepsin-(B + L) and cathepsin-B were assayed as described by Barrett and Kirschke (14). Briefly, 1 μg of each cell lysate was incubated at 37°C for 6 min with the substrates. Free aminomethylcoumarin was measured fluorometrically (excitation at 370 nm, emission at 460 nm) with a fluorometer (F-2000; Hitachi, Ltd, Hitachi, Japan). In order to examine the cathepsin-L activity, the cathepsin-B inhibitor CA-074 (final concentration: 1 μM) was added to cell cultures 60 min before collecting samples and measured cathepsin-(B + L) activity.

Statistical analyses

All quantitative data were presented as mean of three independent experiments ± standard error. Data were analyzed by using a statistical program, Statview 5.0 software for Macintosh computers (Abacus Concepts, Inc., Berkeley, CA, USA). Statistical comparison of two values was done by an unpaired Student's *t*-test. *p*-value less than 0.05 was considered statistically significant.

Results

Effects of long-term cyclosporin A exposure on cell proliferation

As shown in Fig. 1, there is no significant difference in cell proliferation between cyclosporin A-treated cells and non-treated cells ($p = 0.0892$). Among three donors, the same tendency was observed in cell proliferation by long-term cyclosporin A exposure. This result indicates that long-term cyclosporin A exposure had no effect on proliferation of human gingival fibroblasts.

Effects of long-term cyclosporin A exposure on mRNA accumulation of protease-related molecule

The effects of long-term cyclosporin A exposure on mRNA accumulation of MMP-1, TIMP-1, cathepsin-B, -H and -L, and *N*-acetyl-β-D-glucosaminidase

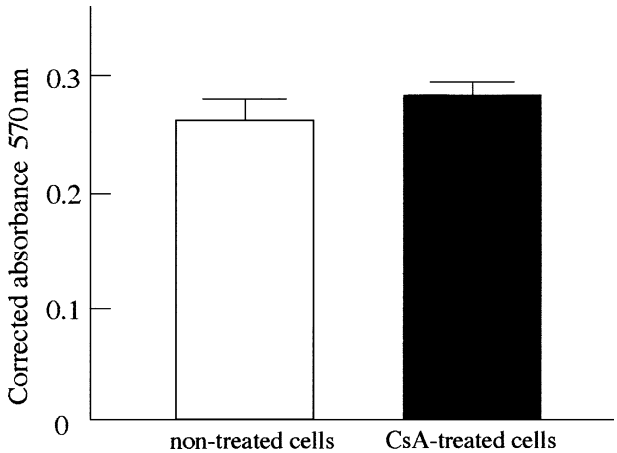

Fig. 1. Long-term cyclosporin A exposure had no effect on proliferative activity. Human gingival fibroblasts were cultured for 5–8 passages with or without 200 ng/ml cyclosporin A. Proliferative activity in human gingival fibroblasts was measured at 8 passage using MTT assay as described in 'Materials and Methods'. Representative findings of three donors are expressed as the mean ± SE of triplicate assays. No significant difference was observed between cyclosporin A-treated cells and untreated cells (Student's *t*-test). CsA, cyclosporin A.

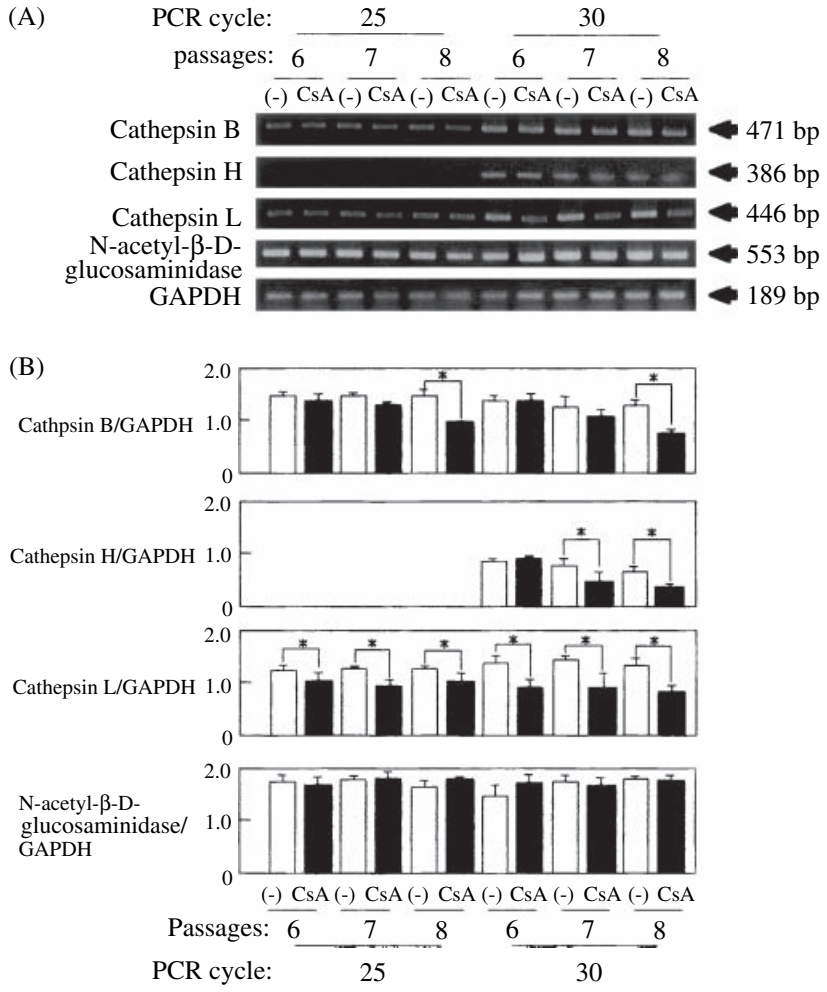

Fig. 2. Effect of Long-term cyclosporin A exposure on accumulation of lysosomal enzymes mRNA. Human gingival fibroblasts were cultured for 5–8 passages with or without 200 ng/ml cyclosporin A. (A) Total RNA was isolated at each time point of 6–8 passages and an aliquot of the reverse-transcribed cDNA was used for semi-quantitative reverse transcription-polymerase chain reaction analysis as described in 'Materials and Methods'. Typical data of representative findings from three donors is shown. (B) The relative intensity of amplified cDNA encoding lysosomal enzymes was calculated and expressed as a ratio against that encoding GAPDH. Values are shown as means ± SE from three donors' samples. $*p < 0.05$ (Student's *t*-test). CsA, cyclosporin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.

were examined using semi-quantitative RT-PCR. As shown in Fig. 2, accumulation of lysosomal enzymes cathepsin-B, -H and -L mRNAs of cyclosporin A-treated cells was markedly suppressed, compared to non-treated cells (passages 8, 25 cycles, cathepsin-B: $p = 0.0043$; cathepsin-L: $p = 0.0027$; passages 8, 30 cycles, cathepsin-B: $p < 0.0001$; cathepsin-H: $p = 0.0056$; cathepsin-L: $p = 0.0089$). Accumulation of the other lysosomal enzyme *N*-acetyl-β-D-glucosaminidase mRNA was not apparently impaired (passages 8, 25 cycles, $p = 0.2475$; passages 8, 30 cycles, $p = 0.1781$). Interestingly, accumulation of cathepsin-L mRNA was rapidly suppressed at six passages (25 cycles, $p = 0.0040$; 30 cycles, $p = 0.0027$), whereas accumulation of cathepsin-B mRNA was barely suppressed at eight passages (passages 7, 25 cycles, $p = 0.2085$; passages 7, 30 cycles, $p = 0.0692$). Among three donors, although the same tendency was observed in the change of cathepsins mRNA accumulation, we found cathepsin-B mRNA accumulation was entirely impaired by long-term cyclosporin A exposure in human gingival fibroblasts at eight passages obtained from one donor (data not shown). Accumulation of MMP-1 and TIMP-1 mRNAs was not also impaired by long-term cyclosporin A exposure (passages 8, 25 cycles, MMP-1: $p = 0.1547$; TIMP-1: $p = 0.1357$; passages 8, 30 cycles, MMP-1: $p = 0.1318$; TIMP-1: $p = 0.1799$) (Fig. 3). Among three donors, the same tendency was observed in change of MMP-1 and TIMP-1 mRNA accumulation.

Effects of long-term cyclosporin A exposure on cathepsin activity

Long-term cyclosporin A exposure significantly suppressed not only the activity of the active form of cathepsin-(B + L) compared to the activity in non-treated cells ($p = 0.0458$) (Fig. 4A), but also the activity of the active form of cathepsin-B ($p < 0.0001$) (Fig. 4B). To evaluate the effect of long-term cyclosporin A exposure on the activity of the active form of cathepsin-L, we partially inhibited the cathepsin-B activity using a selective cathepsin-B inhibitor CA-074. The reduction of the activity of the active form of cathepsin-(B + L) by CA-074 was statistically suppressed by cyclosporin A ($p = 0.0088$) (Fig. 4A), suggesting that the activity of the active form of cathepsin-L was also suppressed by long-term cyclosporin A exposure. Next, we examined the effects of long-term cyclosporin A exposure on total activity of cathepsin-B and -L. Long-term cyclosporin A exposure significantly suppressed total activity of cathepsin-(B + L) compared to the activity in non-treated cells ($p < 0.0001$) (Fig. 5A). Also, long-term cyclosporin A exposure significantly suppressed total activity of cathepsin-B compared to the activity in non-treated cells ($p < 0.0001$) (Fig. 5B). The reduction of total activity of cathepsin-(B + L) by CA-074 was statistically suppressed by cyclosporin A ($p < 0.0001$) (Fig. 5A), suggesting that total activity of cathepsin-L was also suppressed by long-term cyclosporin A exposure. Importantly, among three donors, the

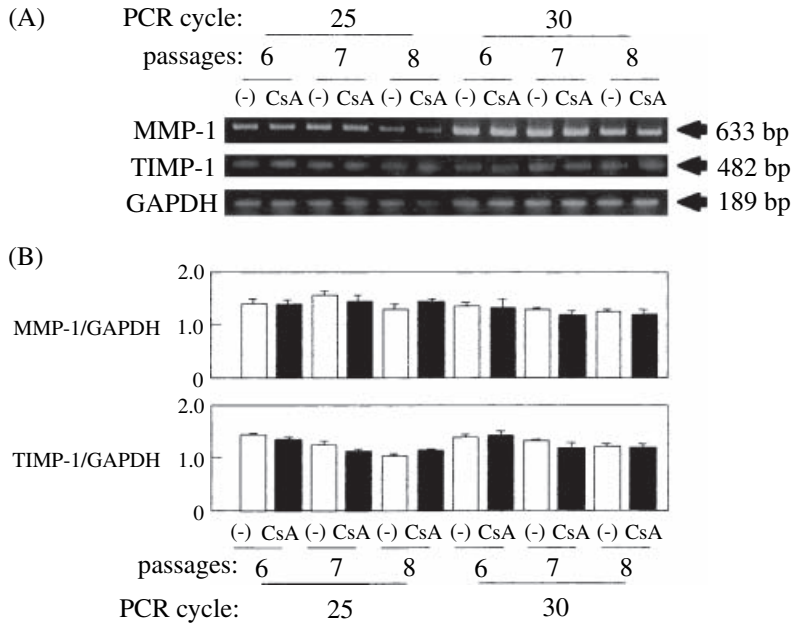

Fig. 3. Long-term cyclosporin A exposure had no effect on accumulation of MMP-1 and TIMP-1 mRNA. Human gingival fibroblasts were cultured 5–8 passages with or without 200 ng/ml cyclosporin A. (A) Total RNA was isolated at each time point of 6–8 passages and an aliquot of the reverse-transcribed cDNA was used for semi-quantitative reverse transcription-polymerase chain reaction analysis as described in 'Materials and Methods'. Typical data of representative findings from three donors is shown. (B) The relative intensity of amplified cDNA encoding MMP-1 or TIMP-1 was calculated and expressed as a ratio against that encoding GAPDH. Values are shown as means ± SE from three donors' samples. CsA, cyclosporin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP-1, matrix metalloprotease-1; PCR, polymerase chain reaction; TIMP-1, tissue inhibitor of matrix metalloprotease-1.

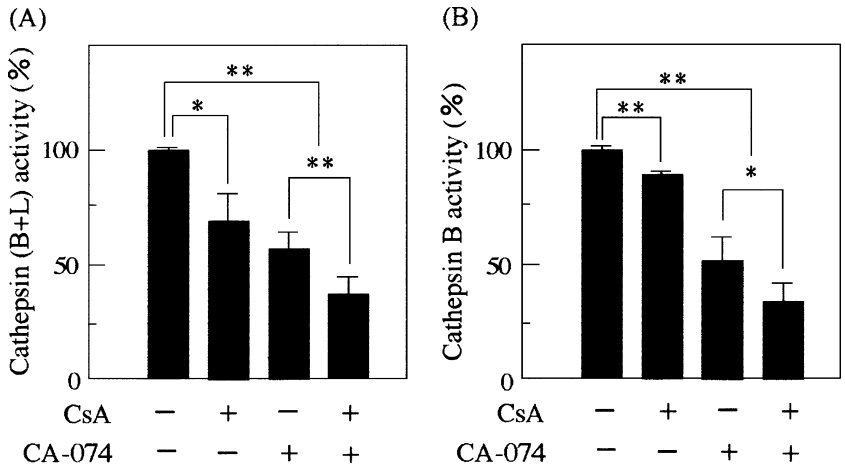

Fig. 4. Long-term cyclosporin A exposure suppresses cathepsin activity (active form). Both cyclosporin A-treated cells and normal cells (8 passages each) were cultured for 1 h with or without CA-074 (1 μM) before sample collecting. Medium was changed daily and substances re-added. Cathepsin activities were expressed relative to the value obtained for untreated cells in each culture series (= 100%). (A) Active form of cathepsin (B + L) activity; (B) active form of cathepsin B activity. Each data represents the mean ± SE of three donors' samples. $*p < 0.05$; $**p < 0.01$ (Student's *t*-test). CsA, cyclosporin A.

same tendency was observed in regard to suppression of cathepsin-B and -L activity by long-term cyclosporin A exposure.

Discussion

The pathogenesis of drug-induced gingival overgrowth may be multifactorial (15, 16). Physiologically, there is a balance between synthesis and degradation of extracellular matrix in gingival connective tissue and this balance must be impaired in overgrown gingiva (17). Although the precise molecular mechanism of gingival overgrowth is not yet clear, several studies have shown the increase of proliferation of matrix-producing cells and subsequent accumulation of matrix proteins (18, 19). On the other hand, it has been described that the pathogenesis of the disease was strongly associated with the increased expression of essential growth factors such as platelet-derived growth factor by accumulated macrophages but not by residual fibroblasts (20).

We have previously shown that cyclosporin A suppressed the activity of cathepsin-L but not cathepsin-B in cultured human gingival fibroblasts (6). In this previous study, confluent cells were treated with 200 ng/ml of cyclosporin A for 0–72 h. However, considering long-term cyclosporin A treatment is indispensable to patients following organ transplantation, it must be important to examine the biological effects of long-term cyclosporin A exposure on human gingival fibroblasts. In the present study, human gingival fibroblasts were cultured for at least three passages with or without cyclosporin A and then the biological effects of long-term cyclosporin A exposure were examined. Leonardi *et al.* reported that a dose-dependent, significant reduction of cell growth was shown with increasing doses of cyclosporin A to more than 10 μg/ml (21). On the other hand, we used a concentration of 200 ng/ml of cyclosporin A, which is within the range of the clinical dose. As shown in Fig. 1, long-term cyclosporin A exposure at this concentration had no effect on the proliferation of human gingival fibroblasts. The discrepancy of

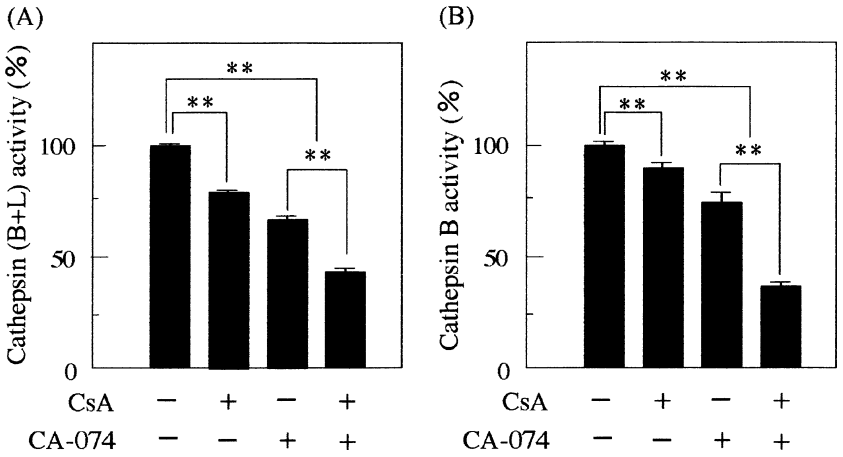

Fig. 5. Long-term cyclosporin A exposure suppresses cathepsin activity (total). Both cyclosporin A-treated cells and normal cells (8 passages each) were cultured for 1 h with or without CA-074 (1 μM) before sample collecting. Medium was changed daily and substances re-added. Before measurement of the activity, pepsin (50 μg/ml) was added to each sample. Cathepsin activities were expressed relative to the value obtained for untreated cells in each culture series (= 100%). (A) total cathepsin (B + L) activity; (B) total cathepsin B activity. Each data represents the mean ± SE of three donors' samples. $*p < 0.05$; $**p < 0.01$ (Student's *t*-test). CsA, cyclosporin A.

the proliferation of human gingival fibroblasts in these studies may be caused by the difference in concentration of cyclosporin A used in the experiments. In addition, although we cannot deny the possibility that some functions of cyclosporin A might be masked by 10% fetal calf serum, we demonstrated that cyclosporin A significantly suppressed cathepsin-B and -L activity (Figs 4 and 5). These results indicate that cyclosporin A used in this study was effective to cultured human gingival fibroblasts, even in the presence of 10% fetal calf serum.

Lysosomal cysteine proteases, cathepsin-B, -H and -L play an important role in type I collagen degradation (5). Cathepsins play an important role in metabolism of gingival collagen fibers because type I collagen is dominant in gingival connective tissue (19). At first, we examined the effects of long-term cyclosporin A exposure on cathepsins mRNA accumulation. As shown in Fig. 2, we demonstrated that cathepsin-B, -H and -L mRNA was impaired by long-term cyclosporin A. Interestingly, we found that cathepsin-B mRNA accumulation was entirely impaired by long-term cyclosporin A exposure in human gingival fibroblasts obtained from one donor (data not shown). In the other two donors, the same tendency was observed in impairment of cathepsins mRNA. This discrepancy of responsiveness to cyclosporin A among three donors may contribute to the difference of development of cyclosporin A-induced gingival overgrowth. Next, we demonstrated that long-term cyclosporin A exposure significantly suppressed the active form of both cathepsin-B and -L activity (Fig. 4). Furthermore, long-term cyclosporin A exposure significantly suppressed total activity of both cathepsin-B and -L (Fig. 5), consistent with its mRNA impairment (Fig. 2). Among three donors, the same tendency was observed in suppression of cathepsin-B and -L activity by long-term cyclosporin A exposure. These results indicate that the effects on human gingival fibroblasts of long-term cyclosporin A exposure are different from the effects of short-term cyclosporin A exposure. We are interested in the discrepancy between the activity of cathepsins and short-term and long-term cyclosporin A exposure, and further experiments are needed to clarify the molecular mechanisms. Interestingly, we found that there was a little difference to the base line of the cathepsin-B and cathepsin-L activity among three cell lines. If the differences of the base line among patients correlate to the pathophysiology of cyclosporin A-induced gingival overgrowth, the concept will contribute to preventive diagnosis in unwanted disease in future. In general, CA-074 binds to cathepsin-B and directly inhibits its activity (22). We demonstrated that reduction of cathepsin-B activity by CA-074 was statistically more suppressed by cyclosporin A (Figs 4 and 5; active form: $p = 0.0342$, total: $p < 0.0001$). Although these results indicate that molecular targets of cyclosporin A could be different from CA-074, we cannot exclude the possibility. This regulation will be subject to further experiments. In addition, we examined the accumulation of *N*-acetyl-β-D-glucosaminidase mRNA. *N*-Acetyl-β-D-glucosaminidase is a main degrading enzyme of keratan sulfate, a glycosaminoglycan (23). This enzyme is secreted by epithelial cells in gingival tissues and contributes to extracellular matrix remodeling in gingival epithelium (24). As shown in Fig. 2, no difference was observed between cyclosporin A-treated cells and non-treated cells in accumulation of *N*-acetyl-β-D-glucosaminidase mRNA. Since one of the histological features of cyclosporin A-induced gingival overgrowth is enlargement of gingival connective tissue, but not that of gingival epithelium, our finding might be a reasonable explanation of the pathogenesis.

Pathophysiologically, clinical validation of specific roles for cathepsin-B and -L proved to be difficult, likely due to overlapping substrate of cysteine-type cathepsins. Cathepsin-B and -L are lysosomal cysteine proteases of broad specificity that are responsible for degradation of structural proteins, such as type I collagen, laminin and proteoglycans (25). In addition, cathepsin-B can degrade fibrinogen, type IV collagen, basement membrane components and fibronectin (26). Furthermore, cathepsin-B can activate latent collagenases, thus playing an indirect role extracellular matrix metabolism (27). Thus, we believe not only cathepsin-L but also cathepsin-B plays various roles in the development of cyclosporin A-induced gingival overgrowth, since impairment of these

enzymatic cascades may be initiated by cathepsin-B suppression.

Recently, it was reported that the expression of TIMP-1 could neutralize the collagenolytic effects of MMP-1 in the pathogenesis associated with pulmonary fibrosis resulting in the accumulation of extracellular matrix (28). This result suggests that the balance of enzymes involved in remodeling of extracellular matrix may affect the development of fibrosis in connective tissue. We therefore examined the effects of long-term cyclosporin A exposure on MMP-1 or TIMP-1 mRNA accumulation in human gingival fibroblasts. Interestingly, we found that long-term cyclosporin A exposure had no effects on their mRNA accumulation (Fig. 3).

From the present study, within the limits of *in vitro* comparisons, cyclosporin A may be considered not only an immunosuppressive agent, but also a direct cathepsin inhibitor in human gingival fibroblasts. Taken together, the suppression of both cathepsin-B and -L activities are, at least, one of factors developing this unwanted side-effect and may ultimately establish a therapeutic concept of this disease in the future.

Acknowledgements

This study was supported by a Grant-in Aid (No. 15209071 to FN) from the Japanese Society for the Promotion of Science.

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