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The upregulation of cystatin C in human gingival fibroblasts stimulated with cyclosporine A

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Background and Objective: Cystatin C is a 13 kDa non-glycosylated, basic protein belonging to the cystatin family. It is consistently and dramatically upregulated in a variety of fibrotic diseases. However, little is known about the correlation between cystatin C and cyclosporine A-induced gingival overgrowth. The aim of this study was to compare cystatin C expression in normal, healthy gingival tissues and cyclosporine A-induced gingival overgrowth specimens and further explore the potential mechanism that may result in cystatin C expression.

Material and Methods: Fifteen cyclosporine A-induced gingival overgrowth specimens and five normal gingival tissues were examined by immunohistochemistry. Three human gingival fibroblast (HGF) strains were established from crown-lengthening surgery. The reverse transcriptase-polymerase chain reaction was used to investigate the effects on HGFs exposed to cyclosporine A. In addition, predominant periodontal pathogens (*Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*) and proinflammatory cytokines (interleukin-1α and tumor necrosis factor-α) were added to seek the possible regulatory mechanisms of cystatin C expression.

Results: The cystatin C staining in gingival tissue was stronger in the cyclosporine A-induced gingival overgrowth group than in the normal gingival group ($p < 0.05$). Intensive staining for cystatin C expression was observed mainly in the cytoplasm of fibroblasts, epithelial cells and inflammatory cells. Moreover, cystatin C expression was significantly higher in cyclosporine A-induced gingival overgrowth specimens with higher levels of inflammatory infiltrates ($p < 0.05$). A concentration of 200 ng/mL cyclosporine A was found to increase cystatin C expression in HGFs in a time-dependent manner ($p < 0.05$). The addition of periodontal pathogens and proinflammatory cytokines significantly increased the expression of cystatin C compared with cyclosporine A alone ($p < 0.05$).

Conclusion: The increased ability of protein accumulation by cystatin C is one of several factors mediating cyclosporine A-induced gingival overgrowth. In addition, cyclosporine A may predispose to gingival overgrowth in inflammatory environments.

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Gingival overgrowth is a common side-effect associated with the systemic use of cyclosporine A, a cyclic endecapeptide, which is an immunosuppressant extensively used in transplant patients to prevent graft rejection, as well as in

the treatment of immunological diseases, such as rheumatoid arthritis and psoriasis (1). Aetiological factors causing and underlying cyclosporine A-induced gingival overgrowth have been reviewed, and it has been demonstrated that local, systemic and genetic factors may also contribute to the development and progression of gingival overgrowth (2,3). Fibroblast proliferation and deposition of excess extracellular matrix (ECM) characterize cyclosporine A-induced gingival overgrowth (4). It is probably a consequence of imbalance between synthesis and degradation of ECM molecules by gingival fibroblasts.

The protein inhibitors of cysteine proteinases, the cystatins, comprise three families: stefins, cystatins and kininogens (5). Cystatins are tight- and reversible-binding inhibitors of the papain-like cysteine proteinases. Cystatin C, one member of the cystatin family, is a non-glycosylated, 13 kDa basic protein that has two disulphide bonds (6) and is a very potent inhibitor of lysosomal cysteine proteinases such as cathepsin-B, -H, -L and -S (7). Cystatin C is consistently and dramatically upregulated in a variety of fibrotic diseases, such as multiple sclerosis sclerosis (8), hepatic fibrosis (9,10) and oral submucous fibrosis (11).

Previous studies have demonstrated that decreased activity of cathepsins may be one of several factors mediating cyclosporine A-induced gingival overgrowth (12,13). In addition, reduced cathepsin-L activity may play an important role in inducing drug-induced gingival overgrowth in cathepsin-L-deficient mice (14). These data suggest that the imbalance between cystatin and cathepsin may play an important role in the pathogenesis of cyclosporine A-induced gingival overgrowth. On the basis of these observations, the present work was undertaken to identify the *in situ* localization of cystatin C expression in normal gingival tissues and cyclosporine A-induced gingival overgrowth specimens. In addition, reverse transcriptase-polymerase chain reactions (RT-PCR) were used to determine the effects of cyclosporine A on the expression of cystatin C in cultured human gingival fibroblasts (HGFs) *in vitro*. The aetiologies of cyclosporine A-induced gingival overgrowth are somewhat controversial because there are different opinions concerning the relative importance of oral hygiene, gingival inflammation and severity of gingival overgrowth (4). Furthermore, prominent periodontal pathogens *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, as well as proinflammatory cytokines interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α), were added to seek the possible regulatory mechanisms of cystatin C expression in an inflammatory environment.

Material and methods

Tissue collection

Normal gingival tissue samples were obtained from five healthy individuals undergoing routine surgical crown lengthening, with little if any evidence of inflammation and no systemic medication. Fifteen redundant hyperplasic gingival biopsies were obtained from ten renal transplant patients receiving cyclosporine A therapy. These patients had been taking cyclosporine A for more than 1 year, and the dose had been adjusted to maintain stable serum levels of about 200 ng/mL. No sign of graft rejection was detected in these renal transplant patients. The samples were obtained during surgical removal of diseased gingiva as part of their routine clinical management, which also included intensive plaque control. Permission for the use of discarded human tissue was obtained from the Institutional Review Board at the Chung Shan Medical University Hospital.

Immunohistochemistry

The surgically removed gingival tissues were fixed in 10% buffered formalin overnight, then dehydrated in an ascending series of graded alcohols and embedded in paraffin. Five-micrometre sections were stained with the monoclonal anti-cystatin C antibody (Santa Cruz Biotechnology, CA, USA; 1:100 dilution) using a standard avidin–biotin–peroxidase complex method (11). 3-Amino-9-ethylcarbazole (Dako, Carpinteria, CA, USA) was then used as the substrate for localizing the antibody binding. Negative controls included serial sections from which either the primary or the secondary antibodies were excluded. The preparations were counterstained with hematoxylin, mounted with Permount (Merck, Darmstadt, Germany) and examined by light microscopy.

One section from each cyclosporine A-induced gingival overgrowth specimen was stained with hematoxylin and eosin to evaluate the magnitude of inflammation at the histological level. Each specimen was graded at ×200 magnification as either 'inflammation low', inflammatory cells < 50% per field or 'inflammation high', inflammatory cells > 50% per field. Grading of each specimen was based on the average inflammatory condition in three consecutive microscopic fields starting from the epithelial–connective tissue border and proceeding gradually deeper into lamina propria.

When processed immunohistochemically for cystatin C expression, sections graded as 'low' were represented by positive-stained cells < 50%; sections graded 'high' exhibited positive stained cells > 50% on three sections from a single tissue sample at ×400 magnification.

Cell culture

Human gingival fibroblasts were cultured by using an explant technique as described previously (15,16). Three healthy individuals were selected from the crown-lengthening procedure for this study. The normal gingival tissue samples were minced using sterile techniques and washed twice in phosphate-buffered saline supplemented with antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL fungizone). Explants were placed into 60 mm Petri dishes and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Gibco Laboratories) and anti-

biotics as described above. Cell cultures between the third and eighth passages were used in this study.

Effect of cyclosporine A on cystatin C mRNA in HGFs

Cells arrested in G_0 by serum deprivation (0.5% FCS for 48 h) were used in the experiments (17). Nearly confluent monolayers of HGFs were washed with serum-free DMEM and immediately thereafter exposed at the indicated incubation times to 200 ng/mL cyclosporine A (Sigma, St Louis, MO, USA). Total RNA was collected at 0, 1, 3, 6 and 24 h for RT-PCR.

Reverse transcriptase-polymerase chain reaction

Total RNA was prepared using TRIzol® reagent (Gibco Laboratories) following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a 15 μL reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukaemia virus reverse transcriptase (Gibco Laboratories). The reaction mixture was diluted with 20 μL of water, and 3 μL of the diluted reaction mixture was used for PCR. The PCR mixture contained 10 pmol of forward and reverse primers and 2 units of Taq DNA polymerase. Amplification was performed using 25 cycles for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and 30 cycles for cystatin C in a thermal cycler. Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 57°C and 1 min of extension at 72°C. The sequences of primers used were as follows (11): GAPDH forward, 5′-TCCTCTGACTTCAACAGCGACACC-3′ and reverse, 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′; and cystatin C forward, 5′-GCTCTTTCCAGATCTACGCT-3′ and reverse, 5′-AGGCAGCCGATGCTACTATT-3′.

The PCR products were analysed by agarose gel electrophoresis, and a 277 bp band for cystatin C was noted. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified in the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

Three replicates of each experiment were performed for each test. All assays were repeated three times to ensure reproducibility. For testing of differences in the cystatin C between normal healthy gingival tissues and cyclosporine A-induced gingival overgrowth specimens, Fisher's exact test was applied for the statistical analysis of the results. The significance of the results obtained from control and cyclosporine A-treated HGFs was statistically analysed by one-way analysis of variance (ANOVA). Tests of differences of the treatments were analysed by Duncan's test. A p-value of < 0.05 was considered to be statistically significant.

Results

Cystatin C expression in normal healthy gingival tissues and cyclosporine A-induced gingival overgrowth specimens is presented in the Table 1. The cystatin C staining in gingival tissue was stronger in the cyclosporine A-induced gingival overgrowth group than in the normal gingival group ($p < 0.05$). Figure 1A represents gingival tissue obtained from normal gingival group with faint cystatin C expression. In the cyclosporine A-induced gingival overgrowth group, intensive red-brown staining for cystatin C expression was observed mainly in the cytoplasm of fibroblasts, epithelial cells and inflammatory cells (Fig. 1B).

Table 1. Cystatin C expression in normal healthy gingival tissues and cyclosporine A-induced gingival overgrowth tissues

	Cyclosporine A-induced gingival overgrowth tissues	Normal healthy gingival tissues
Cystatin C positive	12	1
Cystatin C negative	3	4

Cystatin C expression was found to be significantly higher in cyclosporine A-induced gingival overgrowth tissues than in normal gingival tissues by Fisher's exact test ($p < 0.05$).

Cystatin C expression in cyclosporine A-induced gingival overgrowth specimens with low or high levels of inflammation is presented in Table 2. Differences in cystatin C expression between tissues with low and high levels of inflammation were subsequently analysed using Fisher's exact test. A significantly greater cystatin C expression was noted in cyclosporine A-induced gingival overgrowth tissues with high levels of inflammation ($p = 0.032$).

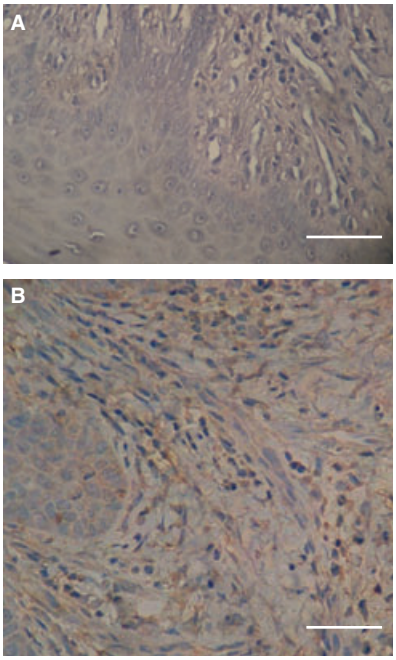

Fig. 1. Immunolocalization of cystatin C in gingival specimens by a peroxidase-labeled streptavidin-biotin technique. (A) Very faint immunoreactivity of cystatin C can be seen in normal human gingival tissues (magnification ×400). (B) Strong immunostaining for cystatin C is present in the cyclosporine A-induced gingival overgrowth specimens. Cystatin C is evident as intensive red-brown coloration in the cytoplasm of fibroblasts, epithelial cells and inflammatory cells (magnification ×400). The bar represents 25 μm.

Table 2. Cystatin C expression and the grade of inflammation in cyclosporine A-induced gingival overgrowth tissues

	Inflammation high	Inflammation low
Cystatin C high	7	1
Cystatin C low	1	4

A significantly greater cystatin C expression was noted in cyclosporine A-induced gingival overgrowth tissues with high levels of inflammation compared with tissues with low levels of inflammatory cell infiltrates by Fisher's exact test ($p = 0.032$).

To examine the effect of cyclosporine A on the cystatin C expression, HGFs were treated with cyclosporine A and the levels of mRNA were measured. The effects of cyclosporine A on the cystatin C gene expression in three different cell strains were similar, and their intracellular variations were limited.

Investigation of the time dependence of cystatin C mRNA expression in 200 ng/mL cyclosporine A-treated HGFs revealed a rapid accumulation of the transcript. A significant signal was first detectable after 1 h of exposure and remained elevated throughout the 24 h incubation period (Fig. 2A). From the AlphaImager 2000, the levels of the cystatin C mRNA increased about 1.4-, 1.8-, 2.6- and 3.1-fold after exposure to cyclosporine A for 1, 3, 6 and 24 h, respectively (Fig. 2B).

Inflammatory mediators were found to increase the cyclosporine A-induced cystatin C expression ($p < 0.05$; Fig. 3A). The quantitative measurement of cystatin C expression by the AlphaImager 2000 is shown in Fig. 3B. *A. actinomycetemcomitans*, *P. gingivalis*, IL-1α and TNF-α were found to elevate cystatin C expression up to 1.9-, 2.0-, 1.4- and 1.9-fold, respectively, compared with cyclosporine A alone ($p < 0.05$).

Discussion

Besides the fact that the increase in ECM is not well understood in cyclosporine A-induced gingival overgrowth, there is no evidence of the expression of cystatin C in this lesion. Only *in vitro* evaluations of lysosomal cysteine proteinases such as cathepsin-B and -L have been published (12,13). Recently, we observed that cystatin C expression was found to be significantly upregulated in the oral submucous fibrosis lesion (11). Proteinase–antiproteinase imbalance may be caused by predominance of proteinases, resulting in severe tissue damage or abundance of proteinase inhibitors, leading to a shift in the balance of synthesis and degradation of ECM proteins and accumulation of these matrix components. It is reasonable to speculate that cystatin C may directly relate to the pathogenesis of cyclosporine A-induced gingival overgrowth.

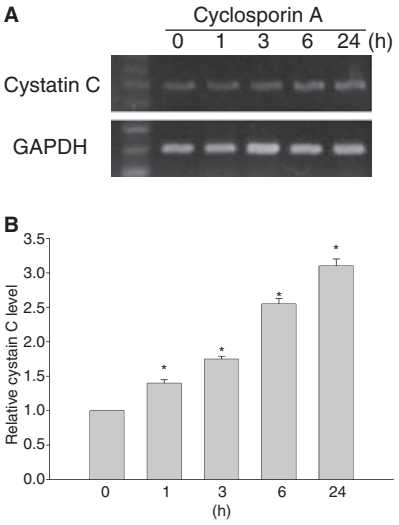

Fig. 2. Expression of cystatin C gene in cyclosporin A- treated HGFs by RT-PCR. (A) Induction of cystatin C mRNA expression by HGFs with 200 ng/mL cyclosporine A. Cells were exposed to cyclosporine A for the indicated times (0, 1, 3, 6 and 24 h). A DNA ladder of known base pairs was used for identification of PCR products. The GAPDH gene was used in order to monitor equal RNA loading. (B) Levels of cystatin C mRNA gene treated with cyclosporine A were measured by AlphaImager 2000. The relative level of PAI-1 mRNA gene expression for each sample was normalized against GAPDH mRNA signal and the control was set as 1.0. Optical density values represent the means ± SD of three different HGF strains. Triplicate experiments were performed. *Significant difference from control values with $p < 0.05$.

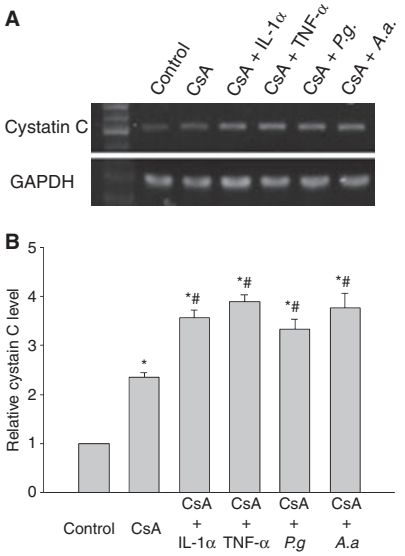

Fig. 3. Various inflammatory mediators were added to seek the possible regulatory mechanism of cystatin C expression. (A) Effects of various inflammatory mediators on cyclosporine A-induced cystatin C mRNA expression in HGFs. Cells were co-cultured with *A. actinomycetemcomitans* (*A.a.*), *P. gingivalis* (*P.g.*), IL-1α, or TNF-α in the presence of 200 ng/mL cyclosporine A. The GAPDH analysis was performed in order to monitor equal RNA loading. (B) Levels of cystatin C mRNA gene treated with cyclosporine A and various inflammatory mediators were measured by AlphaImager 2000. Quantification was achieved by AlphaImager 2000 as described in the legend to Fig. 3(B). Optical density values represent the means ± SD of three different HGF strains. Triplicate experiments were performed. *Significant difference from control values with $p < 0.05$. #Statistically significant difference between cyclosporine A alone and cyclosporine A with various inflammatory mediators; $p < 0.05$.

Cystatin C is a 13 kDa non-glycosylated basic protein belonging to the cystatin family (6). It is consistently and dramatically upregulated in a variety of fibrotic diseases (8–11). To the best of our knowledge, this is the first study to show that cystatin C expression is upregulated in cyclosporine A-induced gingival overgrowth specimens compared with normal gingival tissues. Strong immunostaining for cystatin C was detected in fibroblasts, epithelial cells and

inflammatory cells. In addition, the expression of cystatin C was also shown to increase with the grade of inflammation in cyclosporine A-induced gingival overgrowth specimens. Cystatin C deposition is associated with cyclosporine A-induced gingival overgrowth, suggesting that it could play an important role in the ECM turnover. This suggestion prompted us to propose that cyclosporine A-induced gingival overgrowth may be due to increased synthesis and deposition of ECM proteins, altered degradation or both.

Fibroblasts are the principal cell type residing in connective tissue and are the cells responsible for the formation and turnover of the ECM. Fibroblast function is, in turn, regulated by bioactive molecules acting in the local tissue environment. To the best of our knowledge, this is the first study to show the upregulation of cystatin C mRNA expression in HGFs stimulated by cyclosporine A. This suggests that one of the pathogentic mechanisms of cyclosporine A-induced gingival overgrowth may be the synthesis of cystatin C by resident cells in response to cyclosporine A challenge. The accumulation of ECM components in gingival connective tissue may be caused by a simultaneous effect on cystatin C.

Many studies have suggested that during cyclosporine A treatment, plaque-induced inflammation is associated with the onset or the severity of drug-induced overgrowth (3,18,19), and histological findings have shown the presence of some degree of inflammatory infiltrate in the overgrown gingival tissues (17). In this study, addition of predominate periodontal pathogens and proinflammatory cytokines significantly increased the expression of cystatin C compared with the addition of cyclosporine A alone. Our results differed from those of Fiebiger *et al.* (20), who reported no significant changes in cystatin C levels in response to proinflammatory cytokine in human dendritic cells, and Lautwein *et al.* (21), who found no change in protein expression of cystatin C in human dendritic cells stimulated with lipopolysaccharide. The reason for this contrary result is not clear. It may be a result of the different origins of the test system or different experimental protocols used in each laboratory. However, in the present study, the expression of cystatin C increased with the grade of inflammation in cyclosporine A-induced gingival overgrowth specimens. Taken together, our results suggest that cyclosporine A may predispose to fibrosis via cystatin C overexpression in an inflammatory environment.

The other main extracellular proteolytic systems that have been recognized are the matrix metalloproteinase (MMP)-dependent pathway and the plasminogen-dependent pathway (22). Previous studies have demonstrated that cyclosporine A reduces the secretion of MMPs in gingival fibroblasts (13,23,24). Recently, we found that type I plasminogen activator inhibitor (PAI-1) expression was significantly upregulated in cyclosporine A-induced gingival overgrowth specimens (17). In a previous study, it was shown that transforming growth factor-β could simultaneously increase the activities of MMP and cystatin C in differentiated podocytes (25). Recently, one report has suggested that members of the cystatin family may protect the MMP family from autolytic degradation without interfering with its gelatinolytic activities. In the absence of cystatins, MMPs were completely degraded within 4 h (26). Indeed, the plasmin-dependent pathway is understood to be a significant alternative pathway for the initiation of ECM degradation by MMPs (27). Thus, the regulatory mechanisms may be through several different pathways. The interaction between MMPs, PAI-1 and cystatin C is worthy of further investigation.

As far as we know, this is the first systematic attempt to evaluate the role of cystatin C expression in cyclosporine A-induced gingival overgrowth in humans, both *in vivo* and *in vitro*. We have demonstrated that cystatin C is elevated in cyclosporine A-induced gingival overgrowth tissues compared with normal gingival tissues. Data from our *in vitro* experiments showed that cyclosporine A was capable of stimulating cystatin C mRNA expression in HGFs. This suggests that one of the pathogenic mechanisms of cyclosporine A-induced gingival overgrowth *in vivo* may be the synthesis of cystatin C by resident cells in response to cyclosporine A challenge. In an inflammatory environment, the expression cystatin C can be significantly enhanced in situations where *A. actinomycetemcomitans*, *P. gingivalis*, IL-α or TNF-α levels are raised.

Cysteine proteases secreted or synthesized are inhibited by cystatin on a basis of a ratio of 1:1. Remodelling of ECM is a result of the balance between synthesis and degradation. Recently, a calcium antagonist, nifedipine, was shown to suppress cathepsin-L gene expression and subsequent enzyme activity. Complete loss of cathepsin-L function resulted in the development of gingival overgrowth in mice (14). More detailed studies should be undertaken at both *in vivo* and *in vitro* levels to clarify the roles of cysteine proteases and cystatin in the gingival overgrowth associated with phenytoin, calcium channel blocker or cyclosporine A treatment in humans.

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