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ORIGINAL ARTICLE

Regulation of type I plasminogen activator inhibitor in human gingival fibroblasts with cyclosporine A

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OBJECTIVES: Cyclosporine A (CsA) is used as an immunosuppressive agent and its prominent side effect is the induction of gingival overgrowth. Type I plasminogen activator inhibitor (PAI-1) has shown to play an important role in CsA-induced gingival overgrowth. However, little is known about whether factors can modulate CsA-induced PAI-1 expression.

METHODS: Cytotoxicity, reverse transcriptase-polymerase chain reaction, and enzyme-linked immunosorbent assay were used to investigate the effects of Human gingival fibroblasts (HGFs) exposed to CsA. In addition, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, interlukin-1α, tumor necrosis factor-α, mitogen-activated protein kinase kinase (MEK) inhibitor U0126, signal-regulated protein kinase (ERK) inhibitor PD98059 and cell-permeable glutathione precursor *N*-acetyl-*L*-cysteine (NAC) were added to test how they modulated the effects of CsA-induced PAI-1 expression.

RESULTS: The concentration of CsA higher than 500 ng ml^{-1} demonstrated cytotoxicity to HGFs ($P < 0.05$). Periodontal pathogens as well as proinflammatory cytokines were found to increase the CsA-induced PAI-1 mRNA and protein expression ($P < 0.05$). Pharmacological agents NAC, U0126, and PD98059 were found to decrease the CsA-induced PAI-1 mRNA and protein expression ($P < 0.05$).

CONCLUSIONS: Cyclosporine A (CsA) may predispose to gingival overgrowth under inflammatory environments. The regulation of PAI-1 expression induced by CsA might be critically related with the intracellular glutathione and the ERK-MAPK pathway.

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Introduction

Gingival overgrowth is a common side effect of the chronic use of the immunosuppressive drug cyclosporine A (CsA). The incidence of CsA-induced gingival overgrowth varies from 8 to 85% (Hassell and Hefti, 1991; Pernu *et al*, 1992; King *et al*, 1993). Etiological factors causing and underlying gingival overgrowth have been reviewed and it was determined that local, systemic and genetic factors may also contribute to its development and progression (Marshall and Bartold, 1999; Seymour *et al*, 2000). Histological studies of overgrowth tissues have indicated that an accumulation of extracellular matrix (ECM) within the gingival connective tissue is a central feature of overgrowth tissues (Rostock *et al*, 1986; Mariani *et al*, 1993). Recently, our studies have shown that the upregulation of type I plasminogen activator inhibitor (PAI-1) (Lin *et al*, 2007), lysyl oxidase (Tsai *et al*, 2009a), cystatin C (Tsai *et al*, 2009b), and heat shock protein 47 (Chang *et al*, in press) may contribute to the extracellular components accumulation in CsA-induced gingival overgrowth.

Plasminogen activators and their inhibitors are thought to be key participants in the balance of proteolytic and antiproteolytic activities that regulates ECM turnover. Plasminogen activators in normal plasma and in tissue are inactive and complexed to plasminogen activator inhibitors, of which PAI-1 is believed to be the most important (Vassalii *et al*, 1991; Loskatoff *et al*, 1993). PAI-1 is a single-chain, 50 kDa glycoprotein (Sprengers and Kluft, 1987). PAI-1 is consistently and dramatically upregulated in a variety of fibrotic diseases, including bleomycin-induced pulmonary fibrosis (Barazzone *et al*, 1996), carbon tetrachloride-induced liver fibrosis (Zhang *et al*, 1999), coronary ligation-induced myocardial infarction (Takeshita

et al, 2004) and areca quid chewing associated-oral submucous fibrosis (Yang *et al*, 2003). In addition, bleomycin-induced fibrosis was more severe in transgenic mice overexpressing PAI-1, and less in PAI-1 deficient mice (Eitzman *et al*, 1996).

CsA was found to induce PAI-1 mRNA and protein expression in human gingival fibroblasts (HGFs) (Lin *et al*, 2007). As far as we know, little is known about whether factors can modulate CsA-induced PAI-1 expression. The etiologies of CsA-induced gingival overgrowth are somewhat controversial due to different opinions among the oral hygiene, gingival inflammation and severity of gingival overgrowth (Hassell and Hefti, 1991; Seymour *et al*, 2000). In this study, predominate periodontal pathogens *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) and *Porphyromonas gingivalis* (*P. gingivalis*) as well as proinflammatory cytokines interlukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) were added to seek the possible regulatory mechanisms of PAI-1 expression under inflammatory environment. In addition, mitogen-activated protein kinase kinase (MEK) inhibitor U0126, signal-regulated protein kinase (ERK) inhibitor PD98059, and cell-permeable glutathione (GSH) precursor *N*-acetyl-*L*-cysteine (NAC) were added to find the possible mechanisms and their protective effects.

Materials and methods

Chemicals and materials

CsA, IL-1α, TNF-α, NAC, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). PD98059 and U0126 were obtained from Promega (Madison, WI, USA). All culture materials, TRIzol reagent, and Moloney murine leukemia virus reverse transcriptase were obtained from GIBCO (Grand Island, NY, USA). PAI-1 antigen was purchased from Biopool (Umea, Sweden). CsA, NAC, IL-1α and TNF-α were directly dissolved in the culture medium. PD98059 and U0126 were first dissolved in dimethyl sulfoxide and then diluted with the culture medium. The final concentration of solvent in the medium did not exceed 0.25% (v/v). At these concentrations the solvents used were not cytotoxic to human HGFs. In this study, the final concentration of IL-1α, TNF-α, U0126, PD98059, and NAC used were 10 ng ml^{-1}, 10 ng ml^{-1}, 23 μM, 10 μM, and 1 mM, respectively, as described previously (Chang *et al*, 2004, 2005, 2006; Huang *et al*, 2004).

Bacterial strains and preparation of supernatants

A. actinomycetemcomitans Y4 and *P. gingivalis* (ATCC 33277) were grown under anerobic conditions and harvested at the end of the logarithmic phase of growth as described previously (Chang *et al*, 2002). Briefly, bacterial strains were maintained in brain heart infusion broth, prereduced anerobically sterilized supplemented with hemin (5 mg l^{-1}) and menadione (0.5 mg l^{-1}) for obligate anerobes. The density of the inoculum, prepared in BHI, was adjusted to turbidity of 2 McFarland standard (6×10^8 CFU ml^{-1}). After centrifugation, supernatants were filter-sterilized using a 0.2 μm filter and stored at –80°C until used. The supernatants of *A. actinomycetemcomitans* and *P. gingivalis* were directly diluted in culture medium and the final concentrations of dilution was 1:100.

Cell culture

After approval by the Hospital Review Board, HGFs were cultured by using an explant technique as described previously (Chen *et al*, 2006; Ho and Chang, 2006). Five 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 buffer saline (PBS) supplemented with antibiotics (100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} of fungizone). Explants were placed into 60 mm Petri dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics as described above. Cell cultures between the third and eighth passages were used in this study.

Cytotoxicity assay

The MTT assay is a colorimetric assay based on the ability of the viable cells to reduce a soluble yellow tetrazolium salt (MTT) to blue formazan crystals. MTT solution was prepared as 1 mg ml^{-1} in complete medium just before use. Briefly, 2×10^4 HGFs per well were seeded to 96-well plate and left overnight to attach. Serial dilutions of CsA (0–1000 ng ml^{-1}) in 100 μl volumes were added, and cells were treated for 24 h. After treatment, 50 μl of MTT solution (1 mg ml^{-1} in PBS) was added to each well and incubated for another 4 h at 37°C. To each well, 150 μl of dimethyl sulfoxide was added. Optical density was determined by eluting the dye with dimethyl sulfoxide and the spectrophotometric absorbance measured at 550 nm by using a spectrophotometer (Hitachi, Tokyo, Japan).

Regulation of CsA on PAI-1 mRNA and protein in HGFs

Human gingival fibroblasts (HGFs) arrested in G0 by serum deprivation (0.5% FCS; 48 h) were used in the experiments as described previously (Lin *et al*, 2007; Tsai *et al*, 2009). Nearly confluent monolayers of HGFs were washed with serum-free DMEM and immediately thereafter exposed to 200 ng ml^{-1} CsA. Cultures without FCS were used as negative controls. Subsequently, periodontal pathogens, proinflammatory cytokines and pharmacological agents were added to dishes to test their regulatory effects with 200 ng ml^{-1} CsA. Total RNA was collected at 6 h for reverse-transcriptase polymerase chain reaction (RT-PCR). Condition medium were collected at 24 h for enzyme-linked immunosorbent assay (ELISA).

Reverse-transcriptase polymerase chain reaction

Total RNA was prepared using TRIzol reagent 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 leukemia virus reverse transcriptase. The reaction mixture was diluted with 20 μl of water and 3 μl of the diluted reaction mixture was used for the PCR. PCR reaction mixture contains 10 pmol of forward and reverse primers and 2 units of Tag DNA polymerase. Amplification was performed at 25 cycles for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and 30 cycles for PAI-1 in a thermal cycle. 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 (Lin *et al*, 2007):

1. GAPDH	Forward: 5′-TCCTCTGACTTCAACAGCGACACC-3′
	Reverse: 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′
2. PAI-1	Forward: 5′-ATCACCATCTTCCAGGAG-3′
	Reverse: 5′-ATCACCATCTTCCAGGAG-3′

The PCR products were analyzed by agarose gel electrophoresis and a 325 bp band for PAI-1 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 by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)
Levels of PAI-1 antigen were determined by ELISA. Briefly, 20 μl of conditioned media were directly transferred to the microtest strip wells of the ELISA plate. All further procedures were performed following the manufacturer's instructions. The absorbance at 495 nm was measured in a microtest plate spectrophotometer and PAI-1 levels were determined with a calibration curve using human PAI-1 as a standard. Each value was expressed as the mean $\pm$ s.d.

Statistical analysis
Three replicates of each pharmacological agent were performed in each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was carried out by one-way analysis of variance (ANOVA). Tests of differences of the treatments were analyzed by Duncan's test and a value of $P < 0.05$ was considered statistically significant.

Results

The cytotoxicity of various concentrations (0–1000 ng ml^{-1}) of CsA on HGFs for 24 h by MTT colormetric assay was shown in Figure 1. It was clear that the treatment of CsA, at concentrations ranging from 0–200 ng ml^{-1}, has no cytotoxic effects to HGFs during 24 h incubation period ($P > 0.05$). However, the concentration higher than 500 ng ml^{-1}, CsA demonstrated a cytotoxic effect on HGFs ($P < 0.05$). The concentration upto 1000 ng ml^{-1}, the cell viability was 78% as compared with control. Clinically, the CsA dose for patient has been adjusted to maintain stable serum levels of about 200 ng ml^{-1}. Thus, this concentration was then applied in all subsequent experiments.

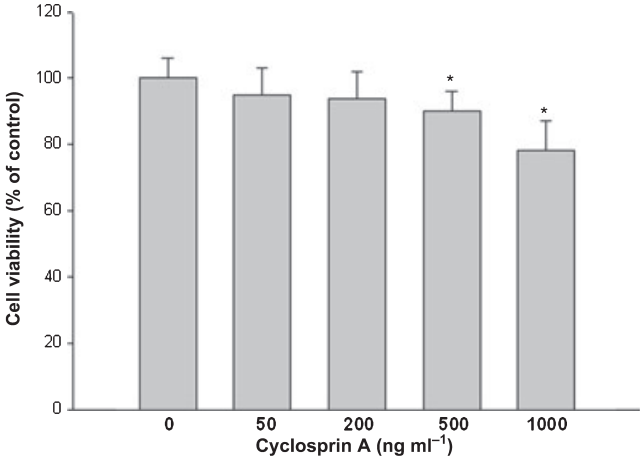

Figure 1 Effects of various concentrations of CsA on cell growth of HGFs. Results are expressed as percentages of absorbance relative to untreated control. Data are shown as the mean of three independent experiments $\pm$ s.d. (bars). *Significant differences from control values with $P < 0.05$

Inflammatory mediators were added to search the possible regulation mechanisms on CsA-induced PAI-1 expression. Periodontal pathogens as well as proinflammatory cytokines were found to increase the CsA-induced PAI-1 mRNA expression (Figure 2). The quantitative measurement of PAI-1 mRNA expression by the AlphaImager 2000 is shown in Figure 3. The levels of the PAI-1 mRNA increased about four-fold after exposure to CsA for 6 h. In addition, CsA with *A. actinomycetemcomitans*, *P. gingivalis*, IL-1α, and TNF-α were found to elevate PAI-1 expression about eight-, five-, 5.2- and six- fold, respectively, as compared with CsA alone ($P < 0.05$).

As shown in Figure 2, pharmacological agents were found to inhibit the CsA-induced PAI-1 expression

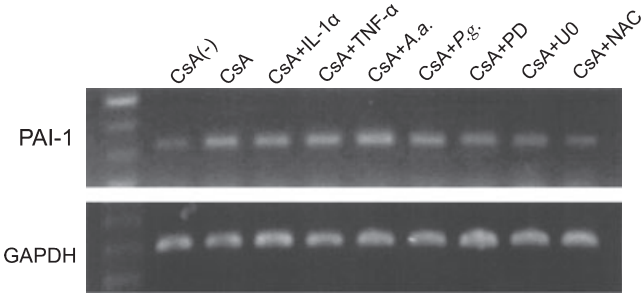

Figure 2 Effects of various inflammatory mediators and pharmacological agents on CsA-induced PAI-1 mRNA expression in HGFs. Cells were co-cultured with *A. actinomycetemcomitans*, *P. gingivalis*, IL-1α, or TNF-α, PD98059, U0126, and NAC in the presence of 200 ng ml^{-1} CsA. GAPDH was performed in order to monitor equal RNA loading

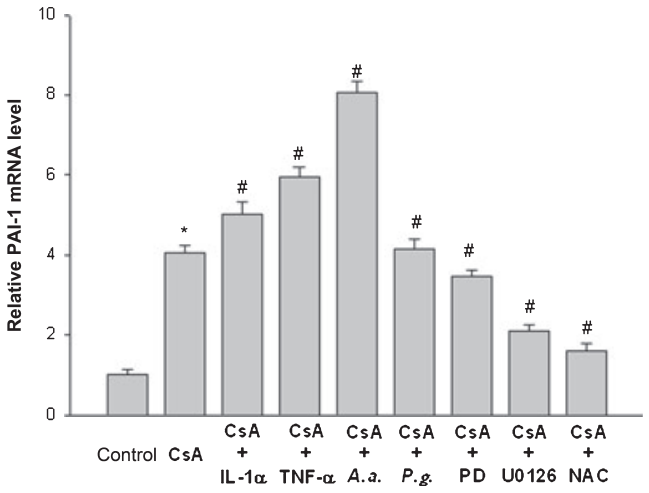

Figure 3 Levels of PAI-1 mRNA gene treated with CsA and various inflammatory mediators and pharmacological agents were measured by AlphaImager 2000. The relative level of PAI-1 expression was normalized against GAPDH mRNA signal and the control was set as 1.0. Optical density values represent the means of five different HGF strains ± s.d. Triplicate experiments were performed. *Significant difference from control values with $P < 0.05$; #statistically significant between CsA alone and CsA with various inflammatory mediators or pharmacological agents; $P < 0.05$

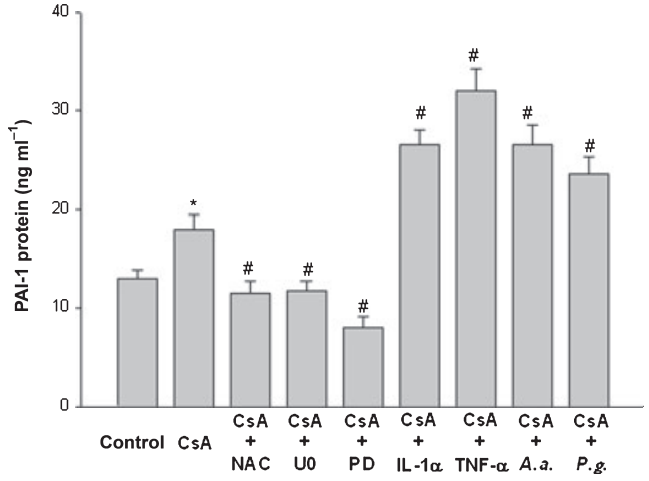

Figure 4 Effects of various inflammatory mediators and pharmacological agents on CsA-induced PAI-1 protein expression in HGFs by ELISA. Cells were co-cultured with *A. actinomycetemcomitans*, *P. gingivalis*, IL-1α, or TNF-α, PD98059, U0126, and NAC in the presence of 200 ng ml^{-1} CsA. *Significant difference from control values with $P < 0.05$; #statistically significant between CsA alone and CsA with various inflammatory mediators and pharmacological agents; $P < 0.05$

($P < 0.05$). The quantitative measurement of PAI-1 mRNA expression by the AlphaImager 2000 is shown in Figure 3. CsA with PD98059, U0126, and NAC were found to significantly decrease the PAI-1 protein expression about 3.4-, 2.2- and 1.4-fold, respectively, as compared with CsA alone ($P < 0.05$).

In addition, the results of RT-PCR were confirmed by ELISA. Similar pattern was seen by RT-PCR. As shown in Figure 4, inflammatory mediators with CsA were found to increase PAI-1 production as compared with CsA alone ($P < 0.05$). Pharmacological agents were found to inhibit the CsA-induced PAI-1 expression ($P < 0.05$). The amounts of PAI-1 protein were about 11.1, 11.3, 7.5, 26.5, 31.5, 26.4, and 23.3 after exposure of CsA with NAC, U0126, PD98059, IL-1α, TNF-α, *A. actinomycetemcomitans*, and *P. gingivalis* for 24 h,respectively.

Discussion

Fibroblasts are the principal cell type residing in connective tissue and 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. In the present study, the viability of cells exposed to CsA (0–200 ng ml^{-1}) was in general cytostatic during 24 h of treatment. The concentration of CsA higher than 500 ng ml^{-1} exhibited cytotoxicity to HGFs. CsA exposure had no effects on cell proliferation. Our results differed from those of several studies which have demonstrated that CsA stimulates the proliferation of HGFs (Mariotti *et al*, 1998; Cotrim *et al*, 2003) and rat gingival fibroblasts (Yoshida *et al*, 2005). However, similar studies were reported previously that CsA had no effects on cell proliferation in lower doses and inhibited cell growth in higher doses in HGFs (James *et al*, 1995; Yamaguchi *et al*, 2004). The reason for this contrary result is not clear. It may be result from different origins of the cells or different experimental protocols used in each laboratory.

The biochemical events involved in the development of the CsA-induced gingival overgrowth are not well understood at present, but the fibrinolytic pathway is believed to play an important role. It is probably a consequence of disturbances in the homeostatic equilibrium between synthesis and degradation of ECM molecules. Recently, our study has shown that PAI-1 expression is significantly upregulated in CsA-induced gingival overgrowth specimens and CsA may be responsible for the enhanced PAI-1 expression *in vivo* (Lin *et al*, 2007). Impaired fibrinolysis may result from increased concentrations of the principal inhibitor of the fibrinolytic system, PAI-1 in CsA-induced gingival overgrowth.

Cyclosporine A (CsA)-induced gingival overgrowth has been associated with poor oral hygiene (Hassell and Hefti, 1991; Seymour *et al*, 2000). It has been reported that bacteria plaque and the resulting gingival inflammation are factors that promote gingival overgrowth significantly (Fu *et al*, 1997). Therefore, it is likely that the mechanism of CsA-induced gingival overgrowth *in vivo* does not only involve direct effects of CsA on HGFs, but also complex interactions with inflammation-promoting constituents of the gingival milieu, such as the constant challenge by bacterial plaque or the presence of inflammatory cytokines. In the present study, the additions of predominate periodontal pathogens *A. actinomycetemcomitans*, *P. gingivalis* and

proinflammatory cytokine IL-1α and TNF-α significantly increased the expression of PAI-1 as compared with the additions of CsA alone. Consistently, the expression of PAI-1 increased with the grade of inflammation in CsA-induced gingival overgrowth specimens (Lin *et al*, 2007). Taken together, our results suggest that CsA may predispose to fibrosis *via* PAI-1 overexpression under inflammatory environment.

As far as we know, the mechanisms and signal transduction pathways involved in the production of PAI-1 within CsA-induced gingival overgrowth are not fully understood. In this study, pharmacological agents were added to search the possible regulation mechanisms on CsA-induced PAI-1 expression. MEK are a unique family of serine/threonine kinases that are activated *via* reversible phosphorylation and mediate signal transduction for multiple extracellular stimuli. PAI-1 expression has been linked with activation of MEK–ERK signaling transduction pathway (Favata *et al*, 1998). To further investigate the mechanism of CsA-induced signaling proteins, MEK inhibitor U0126 and ERK inhibitor PD98059 were added. Both pharmacological agents were found to inhibit CsA-induced PAI-1 expression. Previously, CsA was found to induce ERK activation in HGFs (Chae *et al*, 2006). Consistently, the MEK inhibitor U0126 was found to decrease CsA-induced HSP47 expression in HGFs (Chang *et al*, in press). Therefore, the MEK–ERK signal transduction pathways may be involved in the pathogenesis of CsA-induced gingival overgrowth. The MEK–ERK signal transduction mechanism may be a novel molecular target for the prevention of gingival overgrowth in CsA treated patients.

The most important free radical-removing system in human cells is GSH redox cycle. NAC, a cell-permeable GSH precursor, is easily deacetylated inside the cells and provides cysteine for cellular GSH synthesis and thus stimulates the cellular GSH system (Gillissen and Novak, 1998). In this study, NAC was found to inhibit CsA-induced PAI-1 expression. A previous study has reported that local production of GSH is deficient in patients suffering from pulmonary fibrosis (Cantin *et al*, 1989). Consistently, Wong *et al* (1994) have found that the GSH level is decreased in areca quid chewers with OSF compared with normal buccal mucosa of healthy individuals. Taken together, GSH depletion might play an important role in the pathogenesis of CsA-induced gingival overgrowth. Based on these findings, we propose that use of GSH-rich foods or GSH-like antioxidants may provide a valuable tool in reduction of CsA-induced gingival overgrowth.

In the present study, PAI-1 mRNA and protein expression were enhanced by periodontal pathogens and proinflammatory cytokines. Thus, CsA may predispose to gingival overgrowth under inflammatory environments. PAI-1 expression was inhibited by PD98059, U0126, and NAC. The regulation of CsA induced-PAI-1 expression might be critically related with the intracellular GSH and the ERK-MAPK pathway.

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