Elevated Snail expression in human gingival fibroblasts by cyclosporine A as the possible pathogenesis for gingival overgrowth

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Background/Purpose: Cyclosporine A (CsA) is used as an immunosuppressive agent, and its prominent side effect is the induction of gingival overgrowth. Snail is a master regulator of epithelial–mesenchymal transition (EMT). EMT under pathological processes could lead to fibrotic changes. The purpose of this study was to investigate the role of Snail in the pathogenesis of CsA-induced gingival overgrowth.

Methods: The effect of CsA on normal human gingival fibroblasts (HGFs) was used to elucidate whether Snail expression could be induced by CsA by using quantitative real-time reverse transcription–polymerase chain reaction and western blot. The cell proliferation rate in CsA-treated HGFs with Snail lentiviral-mediated short hairpin RNA interference (shRNAi) knockdown was evaluated by tetrazolium bromide reduction assay.

Results: CsA increased the Snail transcript and Snail protein expression in HGFs in a dose-dependent manner (p < 0.05). In addition, downregulation of Snail by lentiviral infection significantly reduced CsA-stimulated cell proliferation in HGFs (p < 0.05).

Conclusion: CsA stimulated Snail expression and cell proliferation in HGFs, while silencing Snail could effectively reverse these phenomena. These results may provide new avenues for the design of novel antifibrotic therapies for CsA-induced gingival overgrowth through targeting Snail.

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Introduction

Drug-induced gingival overgrowth and fibrosis occurs as a side effect of systemic medication of immunosuppressant cyclosporine A (CsA). CsA-induced gingival overgrowth characterizes fibrosis via the increased accumulation of fibroblasts, collagen, and other extracellular matrix components, such as plasminogen activator inhibitor-1, lysyl oxidase (LOX), cystatin C, heat shock protein 47, and transglutaminase-2. The upregulation of the connective tissue growth factor may contribute to the pathogenesis of CsA-induced gingival overgrowth. In addition, gingival inflammation is a contributing factor to the development of CsA-induced overgrowth, and chlorhexidine was reported to ameliorate gingival overgrowth via an anti-inflammatory mechanism in an established rat model. However, the pathologic mechanisms of CsA-induced gingival overgrowth still need to be further clarified.

Epithelial–mesenchymal transition (EMT) is characterized by the loss of proteins associated with the epithelial phenotype and by the increased expression of proteins associated with a mesenchymal and migratory cell phenotype. EMT occurs in drug-induced gingival overgrowth. Snail, a member of the Snail family of zinc finger transcription factors, is one of the master regulators that promote EMT. Overexpression of Snail is found in various fibrotic diseases, including liver fibrosis and renal fibrosis. However, it is unclear whether Snail is involved in the pathogenesis of CsA-induced gingival overgrowth.

In this study, the effect of CsA on normal human gingival fibroblasts (HGFs) was used to elucidate the possible role of Snail in the pathogenesis of CsA-induced gingival overgrowth. Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) and western blot were used to determine the effects of CsA on the expression of Snail in cultured HGFs in vitro. In addition, the cell proliferation rate in CsA-treated HGFs with Snail lentiviral-mediated short hairpin RNA interference (shRNAi) knockdown was evaluated by tetrazolium bromide reduction assay.

Snail expression analyses

HGFs were arrested in G0 by serum deprivation according to our previous experiments. Nearly confluent monolayers of HGFs were washed with serum-free medium and immediately thereafter exposed at the indicated incubation times to 0 ng/mL, 100 ng/mL, 200 ng/mL, 500 ng/mL, and 1000 ng/mL CsA (Sigma-Aldrich, St. Louis, MO, USA). Cell lysates were collected at 48 hours for qRT-PCR and western blot assays. Cultures without FCS were used as negative control.

qRT-PCR

The total RNA of the cells was purified using a TRizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol as described previously. Briefly, the total RNA (1 μg) of each sample was reverse-transcribed by Superscript II RT (Invitrogen Life Technologies). Then, amplification was carried out in a total volume of 20 μL containing 0.5 μM of each primer, 4 mM MgCl2, 2 μL LightCycler FastStart DNA Master SYBR green I (Roche Molecular Systems, Alameda, CA, USA), and 2 μL 1:10 diluted cDNA. Snail primers were designed: (forward) CTGCCCAATGCTCATCTGGGACTCT and (reverse) ATGACCTTGCC

Snail expression analyses

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were designed: (forward) GGCCCTTTCAGGCCTGGAGAT and (reverse) GTTAA

Western blot assay

Cell lysates were collected as described previously. Briefly, cells were solubilized with sodium dodecyl sulfate solubilizer buffer (5 mM EDTA, 1 mM MgCl2, 50 mM Tris-HCl, pH 7.5 and 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 1 mM N-ethylmaleimide) for 30 minutes on ice. Then, the cell lysates were centrifuged at 12,000 g at 4°C, and the protein concentrations determined with a Bradford reagent using bovine serum albumin as standard. Equivalent amounts of total protein per sample of cell extracts were run on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immediately transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 3% bovine serum albumin for 2 hours, rinsed, and then incubated with primary antibody anti-Snail (1:1000: L70G2, #3895S, Cell Signaling Technology, Inc., Danvers, MA, USA) in PBS containing 0.05% Tween 20 for 2 hours. After three washes with Tween 20 for 10 minutes, the membranes were incubated for 1 hour with biotinylated secondary antibody diluted 1:1000 in the same buffer, washed again as described above, and treated with 1:1000

Methods

Cell culture

After approval by the Institutional Review Board of Chung Shan Medical University Hospital, Taichung, Taiwan, HGFs were cultured by using an explant technique described previously. Three healthy individuals were selected from the crown lengthening procedure for this study. 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, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; GIBCO) and the antibiotics described above. Cell cultures between the third and eighth passages were used in this study.
streptavidin–peroxidase solution for 30 minutes. After a series of washing steps, protein expression was detected by chemiluminescence using an ECL detection kit, and relative photographic density was quantitated by scanning the photographic negatives on gel documentation and analysis system (Alphalager 2000; Alpha Innotech Corp., San Leandro, CA, USA). Each densitometric value was expressed as the mean ± SD.

Snail knockdown in CsA-treated HGFs by lentiviral-mediated shRNAi

The pLV-RNAi vector was purchased from Biosettia Inc. (San Diego, CA, USA). The method of cloning the double-stranded shRNA sequence is described in the manufacturer’s protocol. Lentiviral vectors expressing shRNA that targets human Snail (sh-Snail-1: 5′-AAAAGGACTTCAATCCA GAGTTATGCAATTGACACTCTGGATAGTCC-3′; sh-Snail-2: 5′-AAAAGGTGTGACTAACTATGCAATTGGATCCAATT GCATAGTGTACACCC-3′) were synthesized and cloned into pLV-RNAi to generate a lentiviral expression vector. shRNA that targets luciferase (sh-Luc: 5′-CCGGACTTACCGTGAGTACTTCGAACTCGAGTTCGAAGTACTCAGCGTA-3′) was utilized for an experimental control. Lentivirus production was performed by transfection of a plasmid DNA mixture with lentivector plus helper plasmids (VSVG and Gag-Pol) into 293T cells using Lipofectamine 2000 (Invitrogen Life Technologies). Supernatants were collected 48 hours after transfection and were then filtered; the viral titers were then determined by fluorescence-activated cell sorting at 48 hours post-transduction. Subconfluent cells were infected with lentivirus in the presence of 8 μg/mL polybrene (Sigma-Aldrich). The green fluorescence protein, which was coexpressed in lentivirus-infected cells, was served as a selection marker to indicate successfully infected cells.

Assay for cell proliferation

HGFs were placed in 96-well plates washed with PBS, and the medium was replaced with DMEM for serum starvation overnight. After treatment with CsA (0 ng/mL, 100 ng/mL, 200 ng/mL, 500 ng/mL, and 1000 ng/mL) for 48 hours, cell proliferation was tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma-Aldrich). In the final 4 hours, 50 μL of the MTT solution was added to each well. Only the mitochondria of viable cells can reduce MTT to formazan. The produced insoluble formazan was dissolved with 150 μL of dimethyl sulfoxide to each well. Reduced MTT color reaction was analyzed using a microplate reader set at A560 nm. The optical density values of the experimental groups were divided by the control value and expressed as a percentage of the control.

Statistical analysis

Three replicates 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. Tests of differences of the treatments were analyzed by Duncan’s test, and p < 0.05 was considered statistically significant.

Results

To examine the effect of CsA on Snail expression in vitro, HGFs were treated with CsA, and the levels of transcript and protein were measured by qRT-PCR and western blot analyses. As shown in Figure 1, CsA increased the Snail transcript in HGFs in a dose-dependent manner (p < 0.05). In addition, CsA upregulated Snail protein expression in a dose-dependent manner (p < 0.05; Figure 2A). From the Alphalager 2000, the levels of Snail protein increased about 1.6-, 3.4-, 3.1-, and 3.0-fold after exposure to 100 ng/mL, 200 ng/mL, 500 ng/mL, and 1000 ng/mL CsA, respectively (Figure 2B).

To further investigate whether Snail played a role in maintaining the properties of CsA-treated HGFs, the approach of the loss-of-function of Snail was first

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**Figure 1** Snail mRNA expression was examined by qRT-PCR. Human gingival fibroblasts were exposed for 48 hours in medium containing various concentrations of CsA as indicated. *p < 0.05. CsA = cyclosporine A.
conducted. The downregulation of Snail in CsA-treated HGFs was achieved by viral transduction with lentiviral vector–expressing shRNA targeting Snail. In addition, lentiviral vector–expressing sh-Luc was used as control. qRT-PCR confirmed that lentivirus expressing both sh-Snail markedly reduced the level of CsA-induced Snail expression in HGFs (Figure 3). Immunoblotting analyses confirmed that knockdown of Snail could reduce Snail expression in CsA-stimulated HGFs (Figure 4).

In addition, knockdown of Snail impaired CsA-stimulated proliferation ability in HGFs (Figure 5). In summary, our results suggested that Snail expression may play a major role in CsA-induced proliferation in HGFs.
Elevated Snail expression in human gingival fibroblasts

The biochemical events involved in the development of CsA-induced gingival overgrowth are not well understood at present. EMT is involved in embryonic development in several cellular functions that positively affect tumor development and progression, and fibrosis. Snail is reactivated as a standard model for progressive renal fibrosis in mice and in fibrotic lesions obtained from people who have undergone nephrectomy. In this study, CsA was for the first time found to upregulate Snail mRNA and protein expression in HGFs. In addition, qRT-PCR and immunoblotting analyses confirmed that lentivirus expressing both sh-

Snail-1 and sh-Snail-2 markedly reduced the level of CsA-induced Snail transcript and protein expression in HGFs.

Snail expression may be due to the ROS generation by CsA. These results indicate that snail could play an important role in the pathogenesis of CsA-induced gingival overgrowth.

**Discussion**

The mechanism responsible for Snail expression by CsA may be explained as follows. Snail is triggered by reactive oxygen species (ROS) and upregulated by oxidative stress. CsA could generate ROS in HGFs. Thus, CsA-induced Snail expression may be due to the ROS generation by CsA. These results indicate that Snail could play an important role in the pathogenesis of CsA-induced gingival overgrowth.

**Figure 6** Schematic of the possible mechanisms involved in CsA-induced Snail expression in HGFs. CsA = cyclosporine A; HGF = human gingival fibroblast.

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**Figure 4** The total proteins prepared from a single-cell suspension of human gingival fibroblasts transduced with sh-Luc or sh-Snail lentivirus, individually or concurrently, and treated with or without CsA were analyzed for CsA expression by western blotting. GAPDH was used as protein loading control. The method of measurement is described in Figure 2B. CsA = cyclosporine A; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

**Figure 5** The silencing of Snail expression suppresses the CsA-stimulated cell proliferation capabilities of HGFs. The cell viability of the control and Snail-knockdown CsA-treated HGFs was evaluated by an MTT assay. The cells were exposed for 48 hours in medium containing various concentrations of CsA as indicated. *p < 0.05, sh-Luc + CsA group vs. control group. **p < 0.05, sh-Snail-1 + CsA or sh-Snail-2 + CsA vs. sh-Luc + CsA group. CsA = cyclosporine A; HGF = human gingival fibroblast.

**Figure 6** Schematic of the possible mechanisms involved in CsA-induced Snail expression in HGFs. CsA = cyclosporine A; HGF = human gingival fibroblast.
EMT has an important role in promoting carcinoma invasion and metastasis via enhancing cell adhesion, motility, invasion, and proliferation. Fibroblast proliferation has been characterized in the development and progression of CsA-induced gingival overgrowth. In this study, knock-down of Snail was for the first time found to suppress CsA-stimulated cell proliferation ability in HGFs. This may indicate that the inhibition of the EMT program in HGFs represents a potential antifibrosis therapy for CsA-induced gingival overgrowth.

Snail may play an important role in the pathogenesis of CsA-induced gingival overgrowth. LOX2, a member of the LOX gene family, interacts with Snail and promotes its stabilization by counteracting the action of GSK3β, leading to EMT. Hypoxia-inducible factor-1α (HIF-1α), a key hypoxia-regulated gene, is an important contributor to metastasis and has been shown to induce EMT. LOX and HIF-1α could play an important role in the pathogenesis of CsA-induced gingival overgrowth. In this study, CsA upregulated Snail mRNA and protein expression. Therefore, the interactions among Snail, LOX, and HIF-1α are worthy of further investigation.

As far as we know, this is the first attempt to evaluate the role of Snail expression in HGFs stimulated by CsA. Data from our in vitro experiments showed that CsA stimulated Snail expression and cell proliferation in HGFs, while silencing Snail could effectively reverse these phenomena. Snail may play an important role in the pathogenesis of CsA-induced gingival overgrowth. This study would greatly contribute to a deeper understanding of pathogenesis in CsA-induced gingival overgrowth and promote the development of effective therapies for CsA-induced gingival overgrowth through targeting Snail.

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