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ORIGINAL ARTICLE

Upregulation of embryonic stem cell marker Nanog in human gingival fibroblasts stimulated with cyclosporine A: An *in vitro* study

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KEYWORDS
cyclosporine A;
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Abstract *Background/purpose:* Gingival overgrowth is a common side effect of medication with the immunosuppressant cyclosporine A (CsA). This study proposed to verify whether Nanog, an embryonic stem cell marker, contributes to gingival overgrowth stimulated with CsA in human gingival fibroblasts (HGFs).
Materials and methods: The effect of CsA on HGFs was used to elucidate whether Nanog expression could be induced by CsA using quantitative real-time reverse transcription-polymerase chain reaction and Western blotting. Cell growth in CsA-treated HGFs with Nanog lentivirus-mediated short hairpin RNA interference knockdown was evaluated by tetrazolium bromide reduction assay.
Results: CsA upregulated Nanog transcript in HGFs in a dose-dependent manner ($P < 0.05$). CsA was also shown to increase Nanog protein expression in HGFs in a dose-dependent manner ($P < 0.05$). In addition, downregulation of Nanog by lentiviral infection significantly inhibited CsA-stimulated cell growth in HGFs ($P < 0.05$).
Conclusion: CsA upregulated Nanog expression and cell growth in HGFs, while silencing Nanog effectively reversed these phenomena. Nanog may act as a major switch in the pathogenesis of CsA-induced gingival overgrowth.

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Introduction

Cyclosporine A (CsA) is an immunosuppressant widely used to avoid allograft rejection and treat several immune-related conditions. Unfortunately, gingival overgrowth is one of the significant adverse effects of medication with CsA,[1] with a quoted prevalence of between 8% and 100% in transplant patients.[2] The pathogenesis of CsA-induced gingival overgrowth is still not entirely understood. It is believed that the imbalance of extracellular matrix (ECM) molecules accumulation may contribute to CsA-induced gingival overgrowth.[3–9] Recently, the upregulation of epithelial–mesenchymal transition (EMT) makers Snail[10] and Slug[11] were also found to play an important role in the pathogenesis of CsA-induced gingival overgrowth.

The pluripotency-associated transcription factor Nanog was identified based on its ability to support embryonic stem cell (ESC) self-renewal,[12] and is involved in the maintenance of the undifferentiated state of pluripotent stem cells.[13] Recently, ESC marker Nanog was found in keloid scar[14] and liver fibrosis derived from alcohol-related hepatocellular carcinoma.[15] However, it is unclear whether Nanog 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 Nanog in the pathogenesis of CsA-induced gingival overgrowth. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting were used to determine the effects of CsA on the expression of Nanog in HGFs. In addition, cell growth in CsA-treated HGFs with Nanog lentivirus-mediated short hairpin RNA interference (shRNAi) knockdown was evaluated by tetrazolium bromide reduction assay.

Materials and methods

Cell culture

After approval by the Institutional Review Board at Chung Shan Medical University Hospital, Taichung, Taiwan, normal gingival tissue samples were obtained from three healthy male individuals (mean age, 26.7 years; range, 20–32 years) undergoing routine surgical crown lengthening, with little if any evidence of inflammation and no systemic medication. HGFs were cultured by using an explant technique as described previously.[3,9]

Nanog expression analysis

HGFs were arrested in G_0 by serum deprivation according to our previous experiments.[4,7] 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, 500 ng/mL, and 1000 ng/mL CsA (Sigma–Aldrich, St. Louis, MO, USA). Cell lysates were collected at 24 hours for qRT-PCR and western blot assays. Cultures without fetal calf serum (Gibco BRL, Gaithersburg, MD, USA) were used as negative controls.

qRT-PCR

Total RNA of cells was purified using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) as described previously.[10,11] Nanog primers were designed: (forward) ATTCAGGACAGCCCTGATTCTTC and (reverse) TTTTTGCGACACTCTTCTCTGC. The *GAPDH* housekeeping gene was amplified as a reference standard. *GAPDH* primers were designed: (forward) CATCATCCCTGCCTCTACTG and (reverse) GCCTGCTTCACCACCTTC.

Western blotting

The extraction of proteins from cells and immunoblotting procedure were performed as described previously.[8,9]

Nanog knockdown in CsA-treated HGFs by lentivirus-mediated short hairpin RNA interference

The pLV-RNAi vector was purchased from Biosettia (San Diego, CA, USA). The method of cloning the double-stranded shRNA has been described previously.[10,11] Lentiviral vectors expressing shRNA that targets human Nanog (sh-Nanog-1: 5'-AAAAGCATCCGACTGTAAAGAATTTGGATCCAAATTCTTTACAGTCGGATGC-3'; sh-Nanog-2: 5'-AAAAGCTGTGTGTACTCAATGATTTGGATCCAAATCATTGAGTACACACAGC-3') were synthesized and cloned into pLVRNAi to generate a lentiviral expression vector. shRNA that targets luciferase (sh-Luc: 5'-CCGGACTTACGCTGAGTACTTCGAACTCGAGTTCGAAGTACTCAGCGTAAGTTTTTTG-3') was utilized for an experimental control.

Cell growth

HGFs were placed in 96-well plates, washed with phosphate-buffered saline, and cultured without fetal calf serum for starvation overnight. After treatment with 500 ng/mL CsA for 24 hours, cell growth was tested using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit (Sigma–Aldrich) as described previously.[10,11]

Statistical analysis

Three replicates of each experiment 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 a value of $P < 0.05$ was considered statistically significant.

Results

To examine the effect of CsA on Nanog expression, HGFs were treated with CsA and the levels of transcript and protein were measured by qRT-PCR and Western blotting, respectively. CsA increased Nanog transcription in HGFs in a dose-dependent manner ($P < 0.05$; Figure 1). CsA also upregulated Nanog protein expression in a dose-dependent manner ($P < 0.05$). From the AlphaImager 2000 (Alpha Innotech Corp., San Leandro, CA, USA), the amount of