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ORIGINAL ARTICLE

The modulation of hypoxia-inducible factor-1α/plasminogen activator inhibitor-1 axis in human gingival fibroblasts stimulated with cyclosporine A

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Background/Purpose: The prominent side effect of the immunosuppressive drug cyclosporine A (CsA) is gingival overgrowth. Hypoxia-inducible factor (HIF)-1α regulates a wide variety of profibrogenic genes, which are closely associated with tissue fibrosis. The aim of this study was to compare HIF-1α expression in normal gingival tissues and CsA-induced gingival overgrowth specimens and further explore the potential mechanisms that may lead to induction of HIF-1α expression.

Methods: Fifteen CsA-induced gingival overgrowth specimens and five normal gingival tissues were examined by immunohistochemistry. Western blot was used to investigate the effects of CsA on the expression of HIF-1α in cultured human gingival fibroblasts. The effects of CsA on plasminogen activator inhibitor (PAI)-1 expression were evaluated in environmental hypoxia.

Results: HIF-1α staining in gingival tissue was stronger in CsA-induced gingival overgrowth group than normal gingival group ($p < 0.05$). The expression of HIF-1α was significantly higher in CsA-induced gingival overgrowth specimens with higher levels of inflammatory infiltrates ($p = 0.041$). CsA was found to upregulate HIF-1α protein in a dose-dependent manner

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($p < 0.05$). Hypoxia increased CsA-induced PAI-1 protein expression than normoxic conditions ($p < 0.05$).
Conclusion: These results suggest that HIF-1α expression is significantly upregulated in CsA-induced gingival overgrowth specimens. The activation of HIF-1α may promote fibrogenesis by an increase of PAI-1 expression and a subsequent elevation of extracellular matrix production in gingival tissues.

Introduction

Cyclosporine A (CsA) is a cyclic polypeptide used as an immunosuppressive drug. It is widely used to prevent organ transplant rejection and to treat various immunological diseases. Gingival overgrowth is a prominent side effect associated with the systemic use of CsA.[1] Gingival overgrowth as a clinical outcome presents as an increased gingival volume, including an increased number of cells and a higher level of extracellular matrix (ECM) production.[2] Despite extensive research, the precise mechanism underlying the pathogenesis of CsA-induced gingival outgrowth is still unclear.

A key mediator of cellular responses to low oxygen is hypoxia inducible factor-1 (HIF-1), a heterodimeric transcription factor consisting of a constitutively expressed β subunit (also known as aryl hydrocarbon receptor nuclear translocator) and an O_2-regulated α subunit. In normoxia, the HIF-1α subunit is a short-lived polypeptide that undergoes rapid oxygen-dependent hydroxylation on specific proline and asparagine residues by prolyl hydroxylases. In the presence of oxygen, prolyl hydroxylase domain enzymes hydroxylate HIF-1α and enable interaction with von Hippel-Lindau protein, which results in its ubiquitylation and subsequent proteasomal degradation.[3] HIF-1 also induces expression of profibrogenic genes such as tissue inhibitor of metalloproteinase-1, connective tissue growth factor, and plasminogen activator inhibitor (PAI)-1.[4–6] It is thus likely that by upregulating these profibrogenic factors, HIF-1 accelerates tissue fibrosis. In addition, HIF-1α is consistently and dramatically upregulated in a variety of fibrotic diseases, such as keloid,[7] renal fibrosis,[8] and oral submucous fibrosis.[9]

Recently, HIF-1α was found to be expressed in healthy and diseased periodontal tissues.[10] However, little is known about the correlation between HIF-1α and CsA-induced gingival overgrowth. The present work was undertaken to identify the *in situ* localization of HIF-1α expression in normal gingival tissues and CsA-induced gingival overgrowth specimens. In addition, western blot was used to determine the effects of CsA on the expression of HIF-1α in cultured human gingival fibroblasts (HGFs) *in vitro*. Previous findings have suggested that hypoxia, through HIF-1, could enhance fibrogenesis via factors involved in ECM modification, such as PAI-1.[11] Recently, our study has shown that PAI-1 expression is significantly upregulated in CsA-induced gingival overgrowth specimens.[12] To address the mechanisms underlying the hypoxic regulation of PAI-1 expression, the effects of CsA on HGFs were studied in a hypoxia chamber.

Materials 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 biopsy materials were obtained from 10 renal transplant patients (4 women and 6 men) receiving CsA therapy. The average age of the patients was 52 $\pm$ 12 years. These patients had been taking CsA 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. Institutional Review Board permission at the Chung Shan Medical University Hospital was obtained for the use of discarded human tissue.

Immunohistochemistry

The surgically removed gingival tissues were fixed with 10% buffered formalin overnight, the specimens were dehydrated in an ascending series of graded alcohols and embedded in paraffin. Sections (5 μm thick)_were stained with the monoclonal anti-HIF-1α (sc-10790) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:100 dilution) using a standard avidin-biotin-peroxidase complex method,[13,14] and 3-amino-9-ethylcarbazole (AEC; 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 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 CsA-induced gingival overgrowth specimen was stained with hematoxylin and eosin to evaluate the magnitude of inflammation at the histological level. Most of the inflammatory cells present in the infiltrates represented in these specimens were lymphocytes. Each specimen was graded at 200x magnification as: grade low, inflammatory cells $<$50% per field and grade 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* as described previously.[13,15]

Processed immunohistochemically for HIF-1α expression, sections graded as "low" were represented by positive stained cells >50%; sections graded "high" exhibited positive stained cells <50% on three sections/tissue at 400x magnification according to our previous studies.[16,17]

Cell culture

HGFs were cultured by using an explant technique as described previously.[18] 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 (PBS) supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml of fungizone). Explants were placed into 60 mm Petri dishes and maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Gibco Laboratories) and antibiotics as described above. Cell cultures between the third and eighth passages were used in this study.

Effect of CsA on HIF-1α expression in HGFs by western blot

Cells arrested in G_0 by serum deprivation (0.5% FCS; 48 hours) were used in the experiments.[15] Nearly confluent monolayers of HGFs were washed with serum-free Dulbecco's modified Eagle's medium and immediately thereafter exposed to various concentrations (0, 100, 200, and 500 ng/mL) of CsA (Sigma, St Louis, MO, USA) after 24 h incubation period. Cultures without FCS were used as negative controls. Cells were solubilized with sodium dodecyl sulfate-solubilization buffer (5 mM EDTA, 1 mM $MgCl_2$, 50 mM Tris—HCl, pH 7.5 and 0.5% Trition X-100, 2 mM phenylmethylsulfonyl fluoride, and 1 mM *N*-ethylmaleimide) for 30 minutes on ice. Then, cell lysates were centrifuged at 12,000 *g* at 4°C and the protein concentrations determined with Bradford reagent using bovine serum albumin as standards. Equivalent amounts of total protein per sample of cell extracts were run on a 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 antibodies anti-HIF-1α (1:500) 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 streptavidin-peroxidase solution for 30 minutes. After a series of washing steps, protein expression was detected by chemiluminescence using an ECL detection kit (Amersham Biosciences UK Limited, Amersham, Buckinghamshire, UK), and relative photographic density was quantitated by scanning the photographic negatives on a gel documentation and analysis system (AlphaImager 2000; Alpha Innotech Corp., San Leandro, CA, USA). Each densitometric value was expressed as the mean ± standard deviation.

Establishment of hypoxic condition for PAI-1 expression

Cells growing to about 80% confluence were transferred to a hypoxic chamber with auto purge airlock (NexBiOxy; Unimed Healthcare Inc., Taipei, Taiwan). Environmental hypoxic conditions (1%) were achieved in an airtight humidified chamber and continuously flushed with a gas mixture containing 5% CO_2 and 95% N_2. Maintenance of the desired O_2 concentration was constantly monitored during incubation using a microprocessor-based oxygen controller. Normoxic conditions were defined as 20% O_2, 5% CO_2 at 37°C. The culture period and western blot by using anti-PAI-1 antibody (Santa Cruz Biotechnology) were as described above.

Statistical analysis

Three replicates of each experiment were performed for each test to ensure reproducibility. For testing of differences in the HIF-1α between normal healthy gingival tissues and CsA-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 CsA-treated HGFs was statistically analyzed by Kruskal-Wallis test. Tests of differences of the treatments were analyzed by Duncan's test. A p-value <0.05 was considered to be statistically significant.

Results

HIF-1α staining in gingival tissue was stronger in CsA-induced gingival overgrowth group than normal gingival group ($p < 0.05$). Fig. 1A represents gingival tissue obtained from normal gingival group with faint HIF-1α expression. In CsA-induced gingival overgrowth group, intensive red-brown color staining for HIF-1α expression was observed mainly in the cytoplasm of fibroblasts, epithelial cells, and inflammatory cells (Fig. 1B).

HIF-1α expression in CsA-induced gingival overgrowth specimens with low or high levels of inflammation is listed in Table 1. Differences in HIF-1α expression between tissues with low and high levels of inflammation were subsequently analyzed using Fisher's exact test. A significantly greater HIF-1α expression was noted in CsA-induced gingival overgrowth tissues with high levels of inflammation ($p = 0.041$).

To examine the effect of CsA on the HIF-1α expression, HGFs were treated with CsA and the levels of protein were measured. The effects of CsA on the HIF-1α expression in three different cell strains were similar, and their intracellular variations were limited. Expression of HIF-1α in HGFs challenged with CsA was directly assessed in cell lysates using western blot analysis (Fig. 2A). CsA was found to upregulate HIF-1α protein expression in a dose-dependent manner ($p < 0.05$). From the AlphaImager 2000, the levels of the HIF-1α protein increased about 3.5, 3.0, and 1.4 fold after exposure to 100, 200, and 500 ng/ml CsA, respectively (Fig. 2B).

Previously, CsA was found to increase PAI-I protein expression in HGFs.[12] Hypoxia could enhance fibrogenesis via PAI-1 protein involved in ECM modification. As shown in

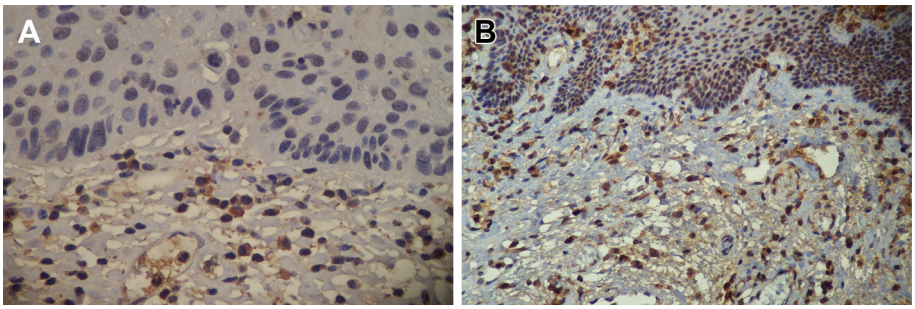

Figure 1 (A) Very faint immunoreactivity of HIF-1α was observed in normal human gingival tissues (400×). (B) Strong immunostaining for HIF-1α was noted in the CsA-induced gingival overgrowth specimens. HIF-1α was evident as intensive redbrown color in the cytoplasm of fibroblasts, epithelial cells and inflammatory cells (200×).

Fig. 3A, CsA was found to upregulate PAI-1 protein expression in a dose-dependent manner under environmental hypoxia and normoxic condition ($p < 0.05$). From the AlphaImager 2000, the levels of the PAI-1 protein increased about 1.3, 2.0, and 2.5 fold after exposure to 100, 200, and 500 ng/ml CsA, respectively, in normoxic conditions (Fig. 3B). In environmental hypoxia, the levels of the PAI-1 protein increased about 2.2, 2.7, and 3.2 fold after exposure to 100, 200, and 500 ng/ml CsA, respectively (Fig. 3B). In addition, CsA-induced PAI-1 protein levels were significantly augmented in environmental hypoxia compared to normoxic condition ($p < 0.05$).

To investigate the axis between HIF-1α and PAI-1 in CsA-induced gingival overgrowth further, HIF-1α inhibitor CAY10585 was utilized in CsA-stimulated HGFs. CsA markedly increased both HIF-1α and PAI-1 protein expression, which was abolished by the addition of CAY10585 under hypoxic condition, respectively (Fig. 4). Taken together, hypoxia through HIF-1α can stimulate PAI-1 accumulation that may enhance CsA-induced gingival overgrowth.

Discussion

Hypoxia plays an important role in regulating a variety of physiological responses as well as in pathological situations. At sites of inflammation, tissue oxygen levels decrease and generate hypoxic stress, which further aggravates and accelerates inflammation and tissue damage.[19] HIF-1α is an important mediator of hypoxia signaling. To the best of our knowledge, this is the first report of HIF-1α expression being upregulated in CsA-induced gingival overgrowth specimens compared with normal gingival tissues. Strong immunostaining for HIF-1α was detected in fibroblasts, epithelial cells, and inflammatory cells. In addition, the expression of HIF-1α was also shown to increase with the grade of inflammation in CsA-induced gingival overgrowth specimens. The reasons are not clear, but it might be explained as follows. Many studies have suggested that the plaque-induced inflammation is associated with the onset or the severity of drug-induced overgrowth,[2] and histological findings have shown the presence of some degree of inflammatory infiltrate in the overgrown gingival tissues.[15,17] In addition, Ng et al[10] have reported that the

Table 1 The results of hypoxia-inducible factor-1α (HIF-1α) expression and the grade of inflammation in cyclosporine A-induced gingival overgrowth tissues.

	Inflammation high	Inflammation low
HIF-1α high	7	1
HIF-1α low	2	5

A significantly greater HIF-1α expression was noted in cyclosporine A-induced gingival overgrowth tissues with high levels of inflammation as compared to tissues with low levels of inflammatory cell infiltrates by Fisher's exact test ($p = 0.041$).

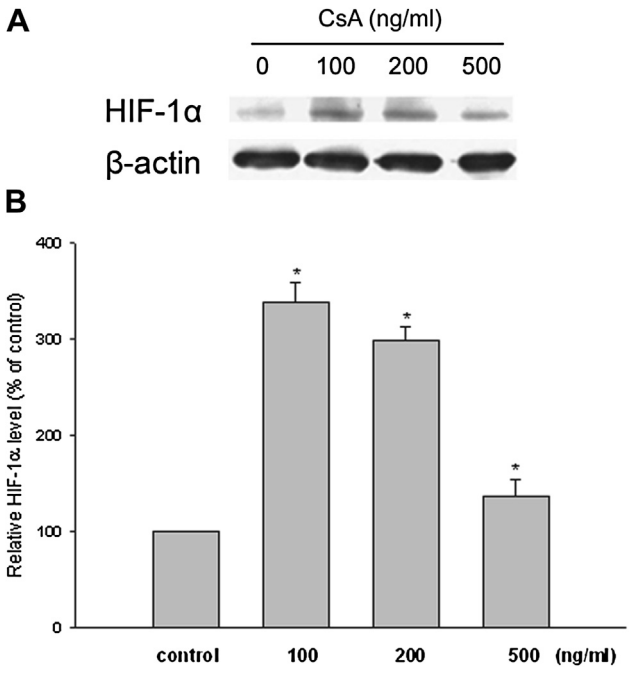

Figure 2 (A) Expression of HIF-1α protein by HGFs in the presence of 0, 100, 200 and 500 ng/ml CsA. Cells were exposed to CsA for a 24 h incubation period. β-actin was performed in order to monitor equal protein loading. (B) Levels of HIF-1α protein treated with CsA were measured by AlphaImager 2000. The relative level of HIF-1α protein expression for each sample was normalized against β-actin signal and the control was set as 1.0. Optical density values represent the means of three different HGF strains standard deviations. Triplicate experiments were performed. * represents significant difference from control values with $p < 0.05$.

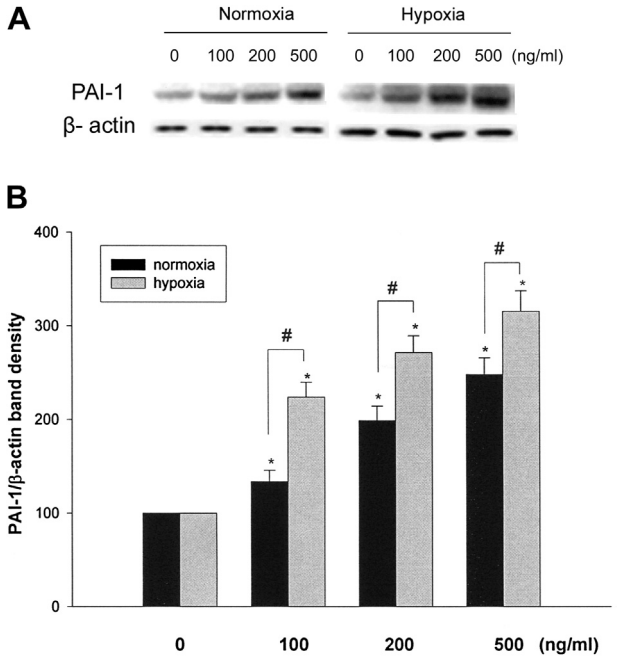

Figure 3 (A) Effects of CsA on PAI-1 protein expression in HGFs under normoxic and hypoxia conditions. (B) The relative level of PAI-1 protein expression for each sample was normalized against β-actin signal and the control was set as 1.0. Optical density values represent the means of three different HGF strains standard deviations. Triplicate experiments were performed. * represents significant difference from control values with $p < 0.05$. # represents statistically significant between normoxic and hypoxia conditions; $p < 0.05$.

marked increase of HIF-1α expression in gingival fibroblasts and infiltrating inflammatory cells in chronic periodontitis implies an important intracellular function of the HIF-1α pathway in periodontal inflammation. Our results suggest that CsA may predispose to fibrosis via HIF-1α overexpression under an inflammatory environment.

HIF-1α is consistently and dramatically upregulated in a variety of fibrotic diseases.[4–6] In this study, we found that the upregulation of HIF-1α expression in HGFs stimulated by CsA. Chronic hypoxia has been newly proposed as a common mechanism of tubulointerstitial fibrosis in the progression of various chronic inflammatory renal diseases,[4,20] where PAI-1 plays an important role in the accumulation of ECM through inhibition of plasmin-dependent ECM degradation. HIF-1α can induce expression of profibrogenic genes such as PAI-1.[11] Recent results suggest that PAI-1 expression is significantly upregulated in CsA-induced gingival overgrowth specimens.[12] We have found that CsA could upregulate PAI-1 protein expression under hypoxia than normoxia. In addition, PAI-1 protein was markedly abolished by HIF-1α inbitior CAY10585 treatment under hypoxic condition. Hypoxic augmentation of the PAI-1 is, at least in part, responsible for the fibrotic phenotype of CsA-induced gingival overgrowth by driving the steady state of ECM metabolism to a state of excessive accumulation. Our data suggest that HIF-1α may exert its profibrotic effect through the upregulation of CsA-induced PAI-1 protein accumulation.

Recently, it has been known that CsA induces transforming growth factor-β1 (TGF-β1) expression in gingival

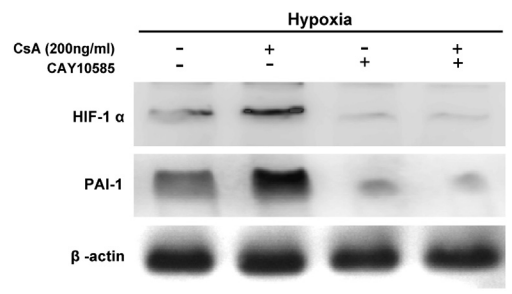

Figure 4 Effects of CsA on PAI-1 and HIF-1α expression in HGFs with or without HIF-1α inhibitor CAY10585 as indicated by western blot.

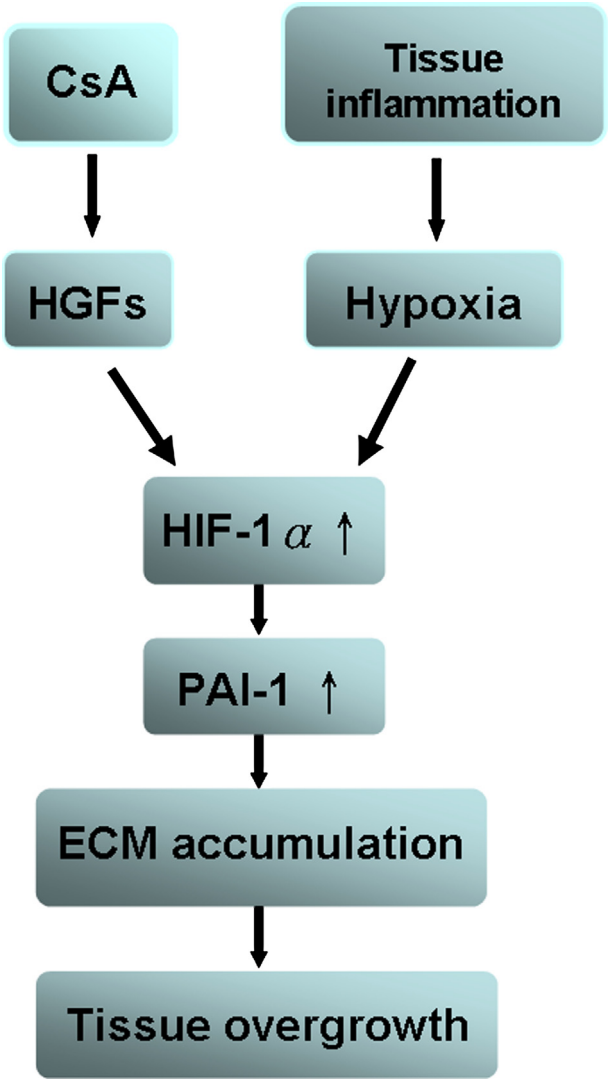

Figure 5 Model proposing a role for HIF-1α/PAI-1 in the progression of CsA-induced gingival overgrowth. Gingival tissue inflammation could generate hypoxic stress and CsA increase HIF-1α expression. As a consequence of hypoxia in HGFs HIF-1α is stabilized, resulting in increased expression of PAI-1, thus promoting the accumulation of ECM.

fibroblasts.[21] HIF-1α was also found to mediate TGF-β-induced PAI-1 production in alveolar macrophages in pulmonary fibrosis.[22] Therefore, the CsA-induced HIF-1α and PAI-1 protein expression in this study could be the effects of CsA-induced TGF-β1 expression. The interaction among TGF-β1, HIF-1α, and PAI-1 is worthy of further investigation.

As far as we known, this is the first systematic attempt to evaluate the role of HIF-1α expression in CsA-induced gingival overgrowth in human at both *in vivo* and *in vitro*. We have demonstrated that HIF-1α is elevated in CsA-induced gingival overgrowth than normal gingival tissues. Data from our *in vitro* experiments show that CsA is capable of stimulating HIF-1α expression in HGFs. Hypoxia through HIF-1α may promote fibrogenesis via stimulation of PAI-1 that promotes ECM accumulation in gingival connective tissues (Fig. 5).

Further research is required, however, including knockout experiments on HIF-1α, using small interfering-RNA for example, specifically to determine whether CsA-induced gingival overgrowth evolves solely as a result of increased/altered *de novo* synthesis and deposition of HIF-1α by CsA. In addition, it would be interesting to know how other HIF-1α regulated profibrogenic factors such as connective tissue growth factor or tissue inhibitor of matrix metalloproteinase-1 will perform in hypoxic conditions.

Acknowledgments

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