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ORIGINAL ARTICLE

Expression of hypoxia inducible factor-1α and vascular endothelial growth factor-C in human chronic periodontitis

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KEYWORDS
hypoxia;
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Abstract *Background/purpose:* Evidence shows that there is a relationship between hypoxia and inflammatory response in periodontitis. Hypoxia-inducible factor (HIF)-1α is a major regulator of energy homeostasis and cellular adaptation to low oxygen stress. Although experimental results demonstrate an association between HIF-1α and vascular endothelial growth factor (VEGF)-C in tumor angiogenesis, the role of HIF-1α and VEGF-C in the pathogenesis of periodontitis is still ambiguous. So far, limited attention has been given to the role of hypoxia and VEGF-C in periodontitis. The present study aimed to investigate the expression and distribution of HIF-1α and VEGF-C in gingival tissue samples from patients with different stages of chronic periodontitis and healthy individuals.
Materials and methods: A total of 56 samples were involved in this study, including moderate chronic periodontitis ($n = 20$), advanced chronic periodontitis ($n = 20$), and healthy control tissues ($n = 16$). The gingival specimens were stained with hematoxylin and eosin for histopathology. The expression of HIF-1α and VEGF-C in gingival tissues was detected by immunohistochemical staining.
Results: HIF-1α and VEGF-C were found in gingival tissues from patients with different stages of chronic periodontitis as well as healthy control tissues. HIF-1α protein was expressed mainly in the epithelial layer of gingival tissues, and VEGF-C protein was mostly located in the connective tissue papilla of gingival tissues. Compared with healthy controls, the expression of HIF-1α and VEGF-C in chronic periodontitis groups was significantly higher ($P < 0.01$), and the density of HIF-1α and VEGF-C in advanced chronic periodontitis group was even significantly higher than that in the moderate chronic periodontitis group ($P < 0.01$).

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Conclusion: Our results suggest that the expression of HIF-1α and VEGF-C increased with severity of periodontitis. So, we conclude that HIF-1α may play an important role in the pathophysiology of human periodontitis and may be related to the function of VEGF-C during periodontitis.

Introduction

Periodontitis is a common chronic infectious disease characterized by the destruction of tooth-supporting tissue, finally leading to tooth loss.[1] In periodontitis, both oxygen supply and demand in the periodontium may be significantly shifted, leading to inflammation-associated tissue hypoxia and metabolic acidosis, disturbing microcirculation and increasing leukocyte infiltration, particularly myeloid cells such as polymorphonuclear leukocytes (PMNs) and monocytes.[2] Low-oxygen tolerance is supported by an adaptive response that includes a coordinate shift in metabolism and the activation of a transcriptional program that is driven by the hypoxia-inducible factor (HIF) pathway.[3] A few affected pathways generally characterize HIF-mediated adaptation responses, including upregulation of angiogenic, erythropoietic, and glycolytic transcripts.[4] Experiments have shown that hypoxia appears to stimulate both innate and adaptive immune responses.[5] Results by Motohira et al[6] showed that hypoxia could stimulate the periodontal ligament cells to produce vascular endothelial growth factor (VEGF), interleukin (IL)-6, and prostaglandin (PG)E_2, which could result in the resorption of alveolar bone in periodontitis.

HIF-1α is a transcription regulatory factor that is encoded by activated gene when organization is under hypoxic.[7] HIF-1α is a major regulator of energy homeostasis and cellular adaptation to low-oxygen stress.[8] HIF-1 functions as a master regulator of cellular and systemic homeostatic response to hypoxia by activating the transcription of many genes, including those involved in energy metabolism, angiogenesis, and apoptosis, and other genes whose protein products increase oxygen delivery or facilitate metabolic adaptation to hypoxia.[9] HIF-1 thus plays an essential role in embryonic vascularization, tumor angiogenesis, and pathophysiology of ischemic disease.[10] At the molecular level, HIF-1α binds to hypoxia response elements (HREs) in the promoters of target genes, such as VEGF and erythropoietin, and promotes their expression.[11] Hypoxia through activation of HIF-dependent transcription of proangiogenic factors such as VEGF is a major mechanism behind tumor angiogenesis. Experiments showed that hypoxia-induced VEGF expression in both neuroblastoma and breast cancer cell lines is primarily driven by HIF-2α at prolonged hypoxia, whereas HIF-1α is the major VEGF inducer during acute hypoxic conditions.[12] So far, knowledge is limited in the role of expression of HIF-1α and VEGF in the pathogenesis of periodontitis.

VEGF produced by many normal and tumor cells plays a key role in regulating normal and abnormal angiogenesis. VEGF is an important growth factor proven to be specific and critical for a mitogen for vascular endothelial cells derived from arteries, veins, and lymphatics, but it is devoid of consistent and appreciable mitogenic activity for other cell types.[13] It binds to endothelial cell surface receptors and activates various functions of the cell including angiogenesis.[14] The periodontal vasculature is affected profoundly during the progression of periodontitis.[15] VEGF primarily stimulates endothelial cell proliferation, chemotaxis, migration, and survival, as well as increasing microvascular permeability and the secretion of proteolytic enzymes.[16,17] VEGF-C increases vascular permeability, which can contribute to the formation of inflammation in the early stages of periodontal disease. VEGF-C seems to be crucial for lymphangiogenesis during periodontal disease development, and upregulation of VEGF-C in recruited immune cells is likely important for the growth of lymphatic vessels.[18] The role of VEGF-C in the pathogenesis of periodontitis is still ambiguous. Although Mkonyi et al[19] have already observed that increased numbers of immune cells expressed VEGF-C in the gingiva after infection, along with upregulation of IL-1β and tumor necrosis factor (TNF)-α at protein levels. On the contrary, Ozcelik et al[20] demonstrated that the expression of VEGF-A and VEGF-C was significantly lower in patients with scleroderma, when compared with the controls. Although higher levels of inflammatory infiltrate and microvessel density were found in the gingival biopsy samples.[20]

Oxygen metabolism has a critical role in maintaining the normal physiological functions of periodontal tissues.[21] During the progression of periodontitis, the status of ischemia and hypoxia in tissue induced the expression of HIF-1α, which can accelerate the expression of VEGF-C. Moreover, VEGF-C is a receptor activator of nuclear factor κB ligand (RANKL) target gene in osteoclasts, and functions as an autocrine factor regulating osteoclast activity. Thus, VEGF-C plays a prominent role in the process of enhancing the resorptive activity of osteoclasts.[22] Therefore, the present study aimed to investigate the expression and distribution of HIF-1α and VEGF-C in human gingival tissues at different stages of chronic periodontitis.

Materials and methods

Gingival tissue collection

The gingival biopsies were obtained from the patients attending the Department of Periodontology, Liwan Stomatological Teaching Hospital of Jinan University,

Table 1 Demographic and pathological data of chronic periodontitis patients and control individuals.

Groups	Total (*n*)	Sex (male/female) *n* (%)	Age (y)		PD (mm)	GI
			Range	Median		
Normal control	16	8/8 (50/50)	25–45	34	—	0.3
Moderate periodontitis	20	8/12 (40/60)	36–63	51	5.4	2.6
Advanced periodontitis	20	11/9 (55/45)	32–65	49	6.3	3.5

GI = gingival index; PD = pocket depth.

Guangzhou, China from September 2011 to September 2013. Fifty-six gingival tissue specimens were obtained from 20 patients with chronic moderate periodontitis, 20 patients with chronic advanced periodontitis, and 16 clinically periodontal healthy individuals. The inclusion criteria were: (1) good health without systemic diseases; (2) no medication was taken at least 2 months ago; (3) no periodontal therapy within 1 year; and (4) pregnant or lactating women were excluded, and postmenopausal women or others under estrogen therapy were excluded. The clinically healthy specimens were harvested from teeth extracted for orthodontic reasons, generally premolars. Specimens of moderate periodontitis were obtained from patients undergoing periodontal surgery, and the specimens of advanced periodontitis were obtained from loose tooth extractions that had severe clinical attachment loss and alveolar bone destruction. All individuals in the moderate and advanced periodontitis groups had previous oral hygiene instruction, scaling, and root planning prior to surgery, but continued to have bleeding on probing from the base of the pocket. Periodontitis were diagnosed according to the 1999 American Academy of Periodontology classification, by measuring gingival index (GI), bleeding on probing (BOP), probing pocket depth (PD), clinical attachment loss (CAL), and by radiography examination.[23] The clinically healthy specimen sites met the following criteria: probing depth not exceeding 3 mm; absence of BOP; GI = 0 or 1; and no radiographic evidence of alveolar bone loss. Patients with moderate periodontitis had gingival inflammation (GI = 2 or 3), BOP, PD, or AL of 4–6 mm and alveolar bone loss of 4–6 mm. Patients diagnosed with advanced periodontitis presented with the following signals: GI = 3 or 4; PD or AL > 6 mm; tooth mobility of Class II or III; alveolar bone loss ≥6 mm. They were 25–65 years old, and 27 patients were male (mean age = 43 years, standard deviation = 11.6) and 29 patients were female (mean age = 42 years, standard deviation = 13.8). The informed consent from the patients obtained prior to the study was approved by the Ethics in Clinical Research Committee of Jinan University. The demographic and pathological data of the patients and healthy individuals included in this study are shown in Table 1. There was no significant difference in age or sex among the three experiment groups.

Histological staining and grading

The collected specimens were fixed in 10% neutral-buffered formaldehyde solution for a minimum of 48 hours before being embedded in paraffin. The 5-μm thick paraffin slides were dewaxed in xylene and rehydrated through a series of graded ethanol, and stained using standard methods with hematoxylin and eosin, and then evaluated for inflammatory changes. The semiquantitative grade of inflammation in gingival tissues was described on a scale from 0 to 3: 0 = no inflammation, no inflammatory cell infiltration; 1 = mild inflammation, local focal inflammatory cells infiltration; 2 = moderate inflammation, spotty and flaky inflammatory cells infiltration; and 3 = severe inflammation, diffuse inflammatory cells infiltration. The grade of inflammation of 10 specimens was measured blindly by two pathologists and the average grade of inflammation was calculated. The sections were visualized and photographed by a Leica DM2500B microscope (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry

Serial paraffin sections (5 μm thick) were treated with conventional dewaxing and hydration. For antigen retrieval, the slides placed in 0.01M citrate buffer solution (pH = 6.0; ZhongShan Goldenbridge Biotechnology, Beijing, China) were heated with high fire for 10 minutes to boil, and with moderate fire for 10 minutes to maintenance in a microwave. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The slides were treated with blocking serum (ZLI-9022; ZhongShan Goldenbridge Biotechnology) for 30 minutes at room temperature. The primary antibodies used in this study were anti-HIF-1α antibody (1:150 dilution; 400080; Millipore Biotechnology, Billerica, Massachusetts, USA) and anti-vegf-C antibody (1:100 dilution; ZA-0266; ZhongShan Goldenbridge Biotechnology). All antibodies were diluted in antibody diluent (ZLI-9028; ZhongShan Goldenbridge Biotechnology). After incubating with blocking serum for 30 minutes, the sections were incubated with primary antibodies overnight at 4°C. The sections were subsequently incubated with goat anti-rabbit immunoglobulin G antibody—horseradish peroxidase multimers (PV-6001; ZhongShan Goldenbridge Biotechnology) for 30 minutes at room temperature, followed by a further coloration with 3,3′-diaminobenzidine (DAB) reagent (ZLI-9017; ZhongShan Goldenbridge Biotechnology) for 8 minutes. Each step was followed by three washes with phosphate-buffered saline for 5 minutes each time. Finally, the sections were counterstained with Mayer's hematoxylin and examined under a light microscope. Negative control staining with no primary antibody was performed by replacing the primary antibody with phosphate-buffered saline, and the other steps were the same as periodontitis groups. Immunohistochemical

staining was evaluated in visual fields at a magnification of 200× under a light microscope.

Assessment of immunohistochemical staining

The slides were analyzed under light microscopy by two pathologists who did not know the clinical diagnosis. The immunohistochemical positive signal was the tiny brown particles that were located in nucleus or cytoplasm. The number of total positive stained cells in gingival tissues was counted in high-power microscopic fields (200×) of each specimen. The size of gingival tissues in each slice was recorded. The density of HIF-1α and VEGF-C positive cells was calculated (number/mm^2). HIF-1α positive cell density (number/mm^2) = total numbers of HIF-1α positive cells of each slice/the tissue size of each slice (mm^2); VEGF-C positive cell density (number/mm^2) = total numbers of VEGF-C positive cells of each slice/the tissue size of each slice (mm^2).

Statistical analysis

All of the periodontal measurements were done in a blind fashion on coded specimens, and the quantitative measurements were made twice. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Nonparametric tests were chosen for variables since the data were not distributed normally. Differences in periodontal histopathological inflammation score and the density of HIF-1α, VEGF-C of different groups were analyzed using the Kruskal—Wallis H test. Two-group comparisons were assessed using Nemenyi test. The relationship between the expression level of HIF-1α and VEGF-C in

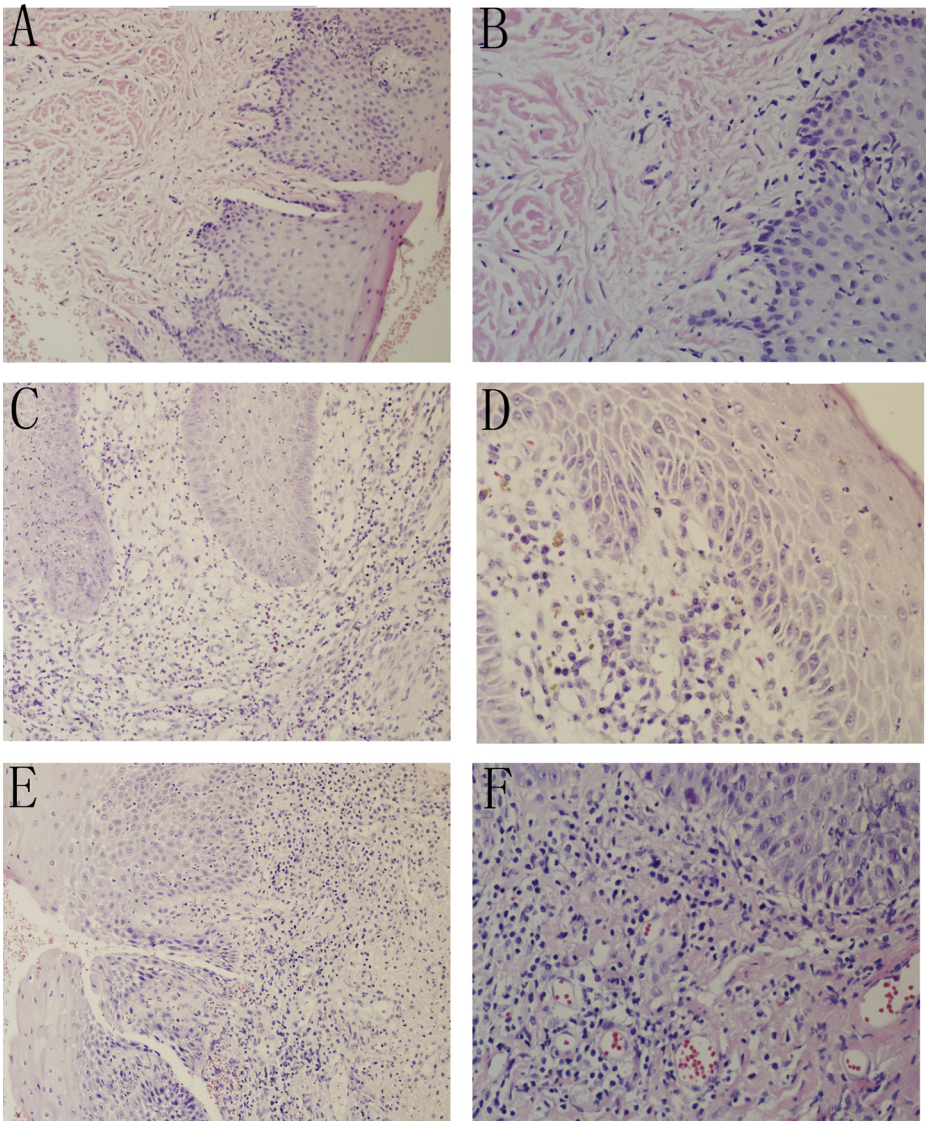

Figure 1 Representative photomicrographs of human periodontal histology stained with hematoxylin and eosin. (A, B) No or slight inflammatory infiltrate in clinically healthy gingival tissues. (C, D) Moderate inflammatory infiltrate in moderate periodontitis group. (E, F) Intense inflammatory infiltrate in advanced periodontitis group. Original magnification: A, C, and E, 200×; B, D, and F, 400×.

gingival tissues was analyzed with Pearson correlation. A P value <0.05 was considered statistically significant.

Results

Histological analysis

Histologically, no inflammatory infiltrate was found in clinically healthy gingival tissues (Figure 1A and 1B). Compared with healthy controls, the specimens of moderate periodontitis showed moderate inflammatory infiltration (Figure 1C and 1D), while advanced periodontitis group presented intense inflammatory cell infiltration (Figure 1E and 1F). The inflammatory scores of each group are shown in Figure 2. Compared with healthy controls, the inflammatory scores of periodontal tissues from both moderate and advanced periodontitis groups were significantly higher ($P < 0.01$). In addition, the inflammatory score of periodontal tissues from the advanced periodontitis group was significantly higher than that of the moderate periodontitis group ($P < 0.01$).

Expression of HIF-1α protein in human gingival tissues

The HIF-1α-positive cells exhibited brown particles in the nucleus or cytoplasm, mainly localized in the epithelial layer of gingival tissues (Figure 3). The density of HIF-1α-positive cells of each group is shown in Figure 4. Compared with healthy controls, the density of HIF-1α-positive cells significantly increased in the periodontitis groups ($P < 0.01$), and the density of HIF-1α-positive cells in the advanced chronic periodontitis group was significantly higher than that of the moderate chronic periodontitis group ($P < 0.01$).

The distribution of HIF-1α-positive cells in human gingival tissues was also evaluated and calculated. The results showed that 96.34 ± 1.80% of HIF-1α-positive cells was located in the epithelial layer of gingival tissues, whereas 3.67 ± 1.80% of HIF-1α-positive cells was located in the connective tissue papilla of gingival tissues. The different ratio of HIF-1α-positive cells in epithelial cell layers and in connective tissues of each group is shown in Figure 5.

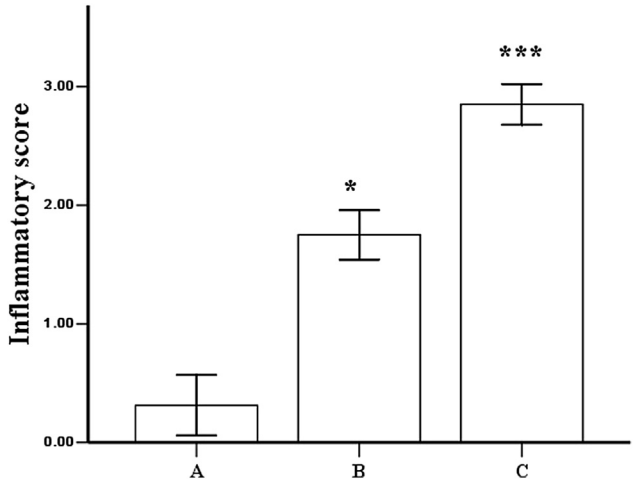

Figure 2 Inflammatory score in the specimen (mean ± SD). (A) Healthy control group ($n = 16$). (B) Moderate periodontitis group ($n = 20$). (C) Advanced periodontitis group ($n = 20$). * $P < 0.01$, compared to healthy control; ** $P < 0.01$, compared to moderate periodontitis. 0 = no inflammation, no inflammatory cell infiltration; 1 = mild inflammation, local focal inflammatory cells infiltration; 2 = moderate inflammation, spotty and flaky inflammatory cells infiltration; 3 = severe inflammation, diffuse inflammatory cells infiltration; SD = standard deviation.

Expression of VEGF-C protein in human gingival tissues

The VEGF-C-positive cells revealed brown particles in the nucleus or cytoplasm, mainly localized in the connective tissue papilla of gingival tissues (Figure 6). The density of VEGF-C-positive cells of each group is shown in Figure 7. Only a few weakly VEGF-C-positive cells were observed in the healthy control group. Compared with the healthy controls, the density of VEGF-C-positive cells was significantly higher in the chronic periodontitis groups ($P < 0.01$), and the density of VEGF-C-positive cells in the advanced chronic periodontitis group was significantly higher than that of the moderate chronic periodontitis group ($P < 0.01$).

The distribution of VEGF-C-positive cells in human gingival tissues was also evaluated and calculated. The results showed that 7.95 ± 5.32% of VEGF-C-positive cells was located in the epithelial layer of gingival tissues, whereas 92.03 ± 5.30% of VEGF-C-positive cells was located in the connective tissues of gingival tissues. The different ratio of VEGF-C-positive cells in epithelial cell layers and in connective tissues of each group is shown in Figure 8.

Negative controls

No cells positive for hypoxia-inducible factor-1α or vascular endothelial growth factor-C were found in negative controls (Figure 9).

Correlation analysis between expression levels of HIF-1α and VEGF-C

Pearson correlation analysis showed that there was a positive correlation between the expression level of HIF-1α and VEGF-C in gingival tissues of healthy controls ($r = 0.963$, $P = 0.000$), moderate periodontitis group ($r = 0.972$, $P = 0.000$) and advanced periodontitis group ($r = 0.987$, $P = 0.000$) (Figure 10).

Discussion

The most common reason for tooth loss is periodontal disease. Until now, the underlying mechanisms involved in periodontal disease have not been fully elucidated. As hypoxia is a common feature of inflamed microenvironments, the moderation of hypoxia may be a significant contributory mechanism in periodontitis.[24] The microenvironment of inflamed and injured tissue in the periodontium

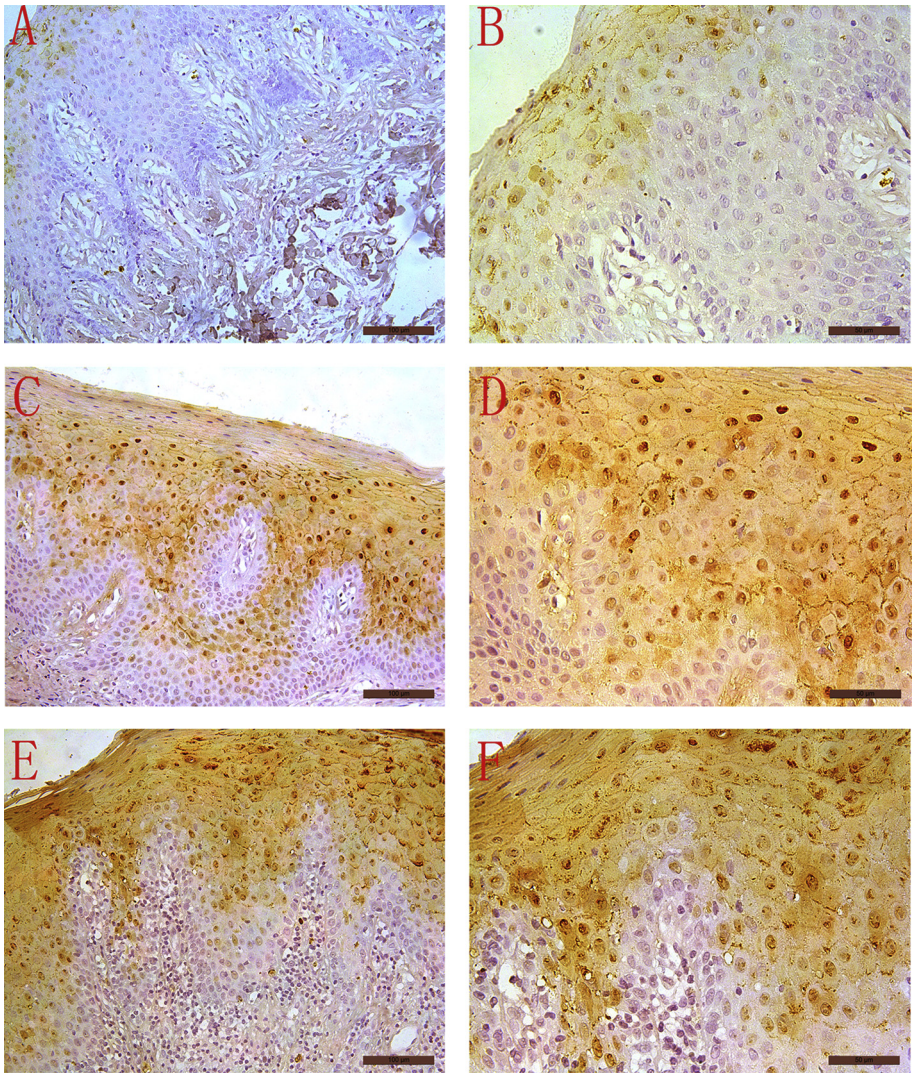

Figure 3 Immunostaining for HIF-1α protein in human gingival tissues. Positively stained cells can be visualized by the brown particles in nucleus or cytoplasm, mostly located in the epithelial layer. There were a few weak positive cells expressing HIF-1α protein observed in the healthy control group (A and B). In the moderate chronic periodontitis group (C and D), the number of HIF-1α-positive cells significantly increased. Numerous cells with strong nuclear or cytoplasmic staining were observed in the advanced chronic periodontitis group (E and F). Original magnification: A, C, and E, 200×; B, D and F, 400×. HIF = hypoxia-inducible factor.

is characterized by low levels of oxygen and glucose, and high levels of inflammatory cytokines, reactive oxygen, and nitrogen species and metabolites. Responses to hypoxia are mediated by HIF protein, a transcription factor that induces genes whose products restore blood supply, nutrients, and energy production to maintain tissue homeostasis.[25] To the best of our knowledge, it is not known whether HIF-1α protein is expressed in gingival tissues of healthy individuals and periodontitis patients. Our results showed that strong expression of HIF-1α was observed in the epithelial layer of gingival tissues in chronic periodontitis, and the increased expression of HIF-1α was related to the enhanced severity of periodontitis; thus, our findings indicated that periodontitis is under anoxic conditions. Hypoxia is a vital element in the pathogenesis of periodontal disease; a previous study showed that there is a correlation of periodontitis severity with psychological stress and periodontal tissue hypoxia.[26]

HIF-1 consists of a constitutively expressed HIF-1β subunit and an oxygen-regulated HIF-1α subunit. Under normal oxygen tension, HIF-1α is rapidly degraded by the ubiquitin—proteasome pathway, however, under hypoxic conditions, HIF-1α is stabilized by the attenuation of proly1 hydroxylase activity.[27] The accumulated HIF-1α heterodimerizes with HIF-1β and translocates into the nucleus. The HIF-1 complex binds to the HRE, composed of a core 5′-ACGTG-3′ sequence, in concert with the transcriptional coactivator p300/CREB-binding protein (CBP),[28] thereby activating the expression of target genes, such as VEGF and erythropoietin.[29] HIF-1α is a major regulator of energy homeostasis and cellular adaptation to low-oxygen stress. Recently, HIF-1α has been discovered to function as a global regulator of macrophage, neutrophil inflammatory, and innate immune functions, as befits these specialized phagocytic cells that operate effectively in the hypoxic microenvironments of infected tissues.[30] In cells of the

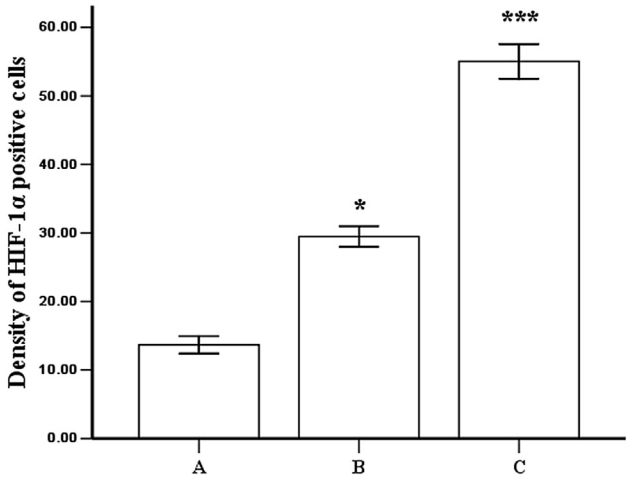

Figure 4 Density of hypoxia-inducible factor-1α positive cells (mean ± standard deviation). (A) Healthy control group ($n = 16$). (B) Moderate periodontitis group ($n = 20$). (C) Advanced periodontitis group ($n = 20$). * $P < 0.01$, compared to healthy control; ** $P < 0.01$, compared to moderate periodontitis.

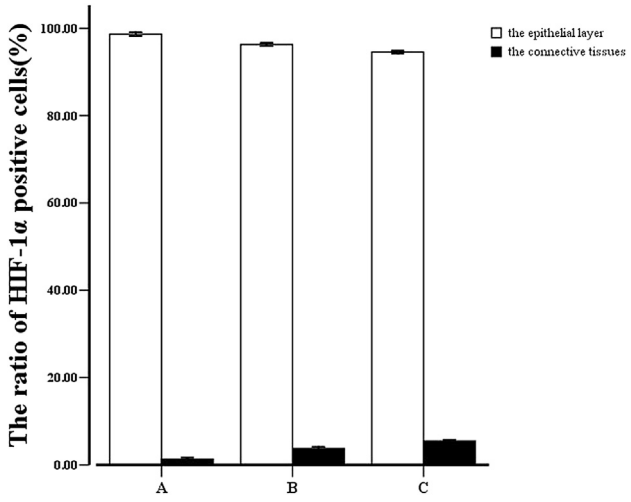

Figure 5 Different ratio of HIF-1α-positive cells in epithelial cell layers and in connective tissues of each group (mean ± standard deviation). The HIF-1α-positive cells were mainly localized in the epithelial layer of gingival tissues. (A) Healthy control group ($n = 16$). (B) moderate periodontitis group ($n = 20$). (C) Advanced periodontitis group ($n = 20$). HIF = hypoxia-inducible factor.

innate and adaptive immune system, HIF-1 is upregulated by bacterial and viral compounds, even under normoxic conditions. This upregulation prepares these cells to migrate, and to function in hypoxic and inflamed tissues. Therefore, HIF-1α protein as a hypoxic marker was chosen to evaluate the hypoxic degree in gingival tissues with periodontitis by immunohistochemistry in this study. To the best of our knowledge, little is known about whether HIF-1 is a potentially important marker for human chronic periodontitis. Ng et al[31] indicated that HIF-1α is expressed in healthy and diseased periodontium. Our results showed similar findings in human gingival tissues with periodontitis. Compared with the healthy controls, immunohistochemistry indicated increased expression level of HIF-1α protein in chronic periodontitis. We also observed that expression of HIF-1α and inflammatory score in advanced periodontitis were significantly higher than those of moderate periodontitis. In particular, increased expression of HIF-1α protein in fibroblast-like cells and infiltrating inflammatory cells in gingival biopsies from patients with chronic periodontitis indicates that the HIF-1 pathway is involved in controlling periodontal inflammatory responses. The increased expression of HIF-lα in some gingival epithelial cells suggests that a steep oxygen gradient may exist between the gingival epithelium and dermal vessels, depending on the diffusion distance, local metabolism, and oxygenated blood supply.[31] Once extravasated from the vasculature, the activity of cells is further enhanced by stimulation of HIF-1 induced by proinflammatory cytokines like IL-1β, TNF-α, and locally expressed tissue factors.[32] Yoshida et al[33] demonstrated that HIF signaling is regulated at post-translational levels via interactions with the master regulator of inflammation NF-κB. NF-κB is a regulator of innate immunity and inflammatory signaling.[33] The RANKL and osteoprotegerin pathway regulates osteoclast differentiation and function during normal physiological bone remodeling, and RANK, RANKL, and osteoprotegerin proteins may play a major role in the bone loss occurring in periodontitis, which are differentially expressed in periodontal tissue.[34]

VEGF-C belongs to a family of heparin-binding growth factors that include VEGF-B, VEGF-C, VEGF-D, and placental-like growth factor.[35] VEGF is a well-known angiogenic factor that is important for vascular development and maintenance in all mammalian organs. The development of molecular tools and pharmacological agents to selectively inhibit VEGF function and block angiogenesis and/or vascular permeability has led to great promise in the treatment of various cancers, macular degeneration, and wound healing.[35] However, VEGF is also important in animals for the regulation of angiogenesis, stem cell and monocyte/macrophage recruitment, maintenance of kidney and lung barrier functions, and neuroprotection.[35] In addition to its role in regulating endothelial cell proliferation, migration, and cell survival, VEGF receptors are also located on many nonendothelial cells and act through autocrine pathways to regulate cell survival and function. VEGF expression is regulated by a variety of stimuli such as nitric oxide, growth factors, and HIF-1α.[36,37] In allergic airway disease, the accumulated data indicated that the tight regulation of HIF-1α activity and VEGF expression is performed by the phosphoinositide 3-kinase/serine threonine kinase/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling cascade.[36] HIF-1 is a key transcription factor in hypoxia-mediated VEGF gene upregulation.[37] VEGF protein binds to its receptor, and these compounds mediate physiological functions. It is well established that the expression of VEGF is upregulated by hypoxia, and the expression of VEGF in response to hypoxia depends on transcriptional activation by a heterodimer comprising HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT)1.[38] HIF-1α binds to HRE of target genes such as VEGF, erythropoietin, and glycolytic enzymes.[39]

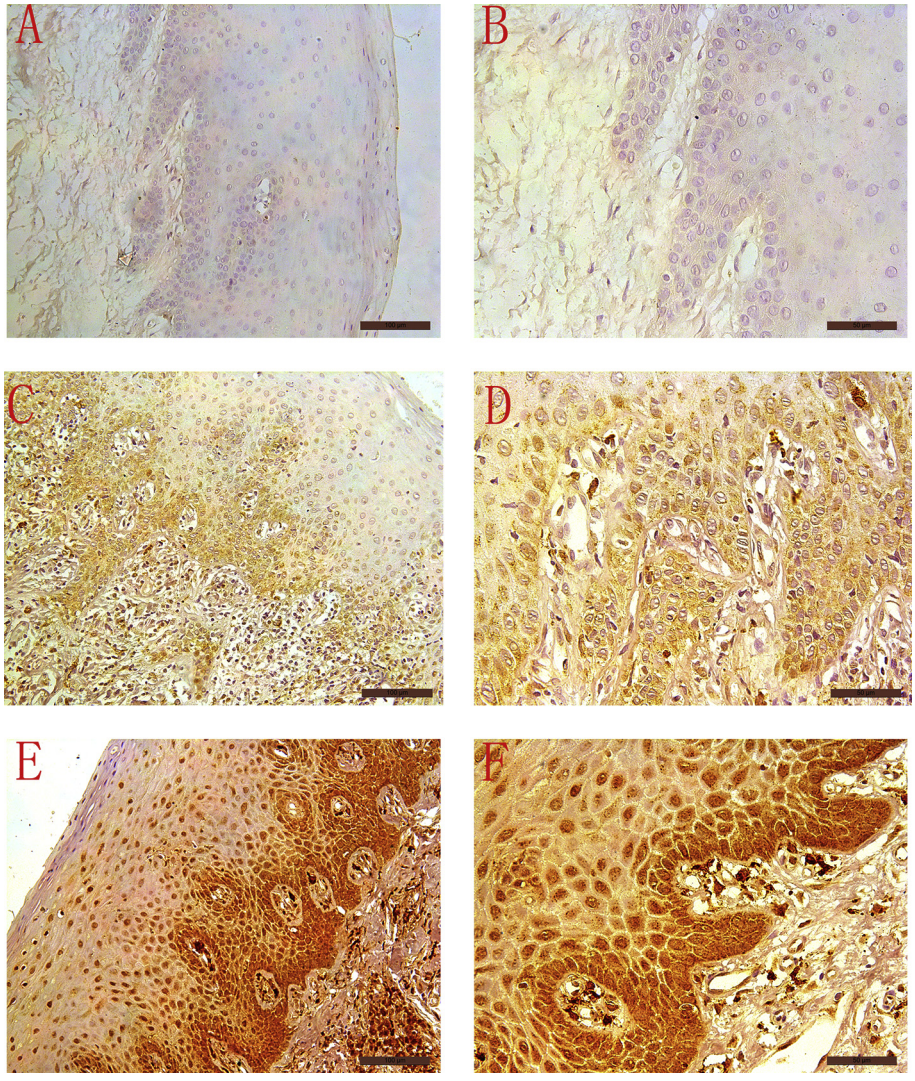

Figure 6 Immunostaining for VEGF-C protein in human gingival tissues. The VEGF-C-positive cells were mainly localized in connective tissue papilla of gingival tissues. There were a few cells weakly positive for VEGF-C protein in the healthy control group (A and B). The number of VEGF-C-positive cells significantly increased in the moderate chronic periodontitis group (C and D). Numerous cells with strong nuclear or cytoplasmic staining were observed in the advanced chronic periodontitis group (E and F). Original magnification: A, C, and E, 200×; B, D, and F, 400×. VEGF = vascular endothelial growth factor.

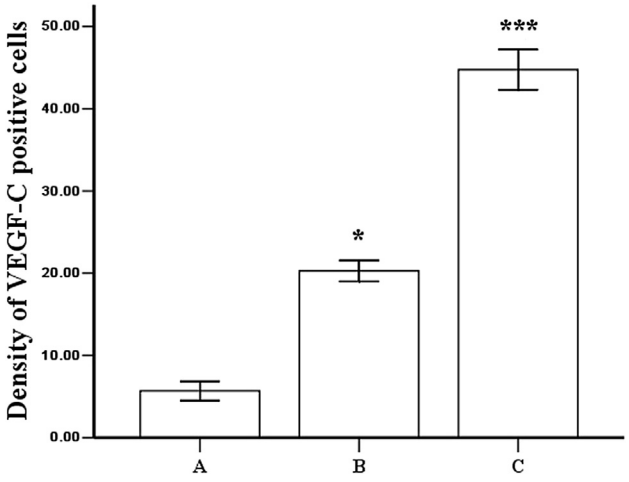

Figure 7 Density of cells positive for vascular endothelial growth factor-C. (A) Healthy control group (n = 16). (B) Moderate periodontitis group (n = 20). (C) Advanced periodontitis group (n = 20). * $P < 0.01$, compared to healthy control; ** $P < 0.01$, compared to moderate periodontitis.

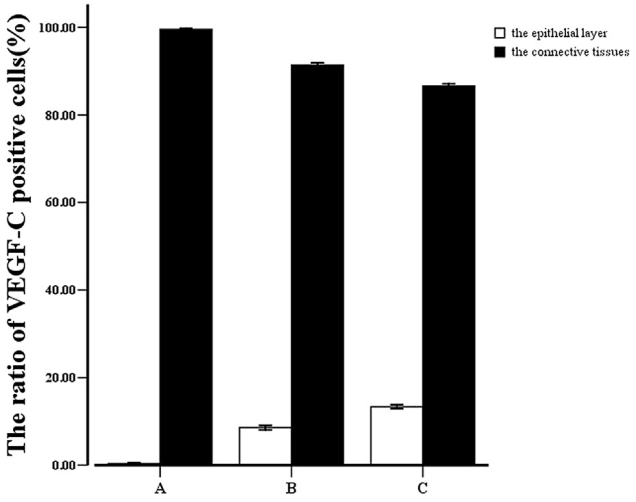

Figure 8 Different ratio of cells positive for vascular endothelial growth factor-C in epithelial cell layers and in connective tissues of each group (mean ± standard deviation). (A) Healthy control group (n = 16). (B) Moderate periodontitis group (n = 20). (C) Advanced periodontitis group (n = 20).

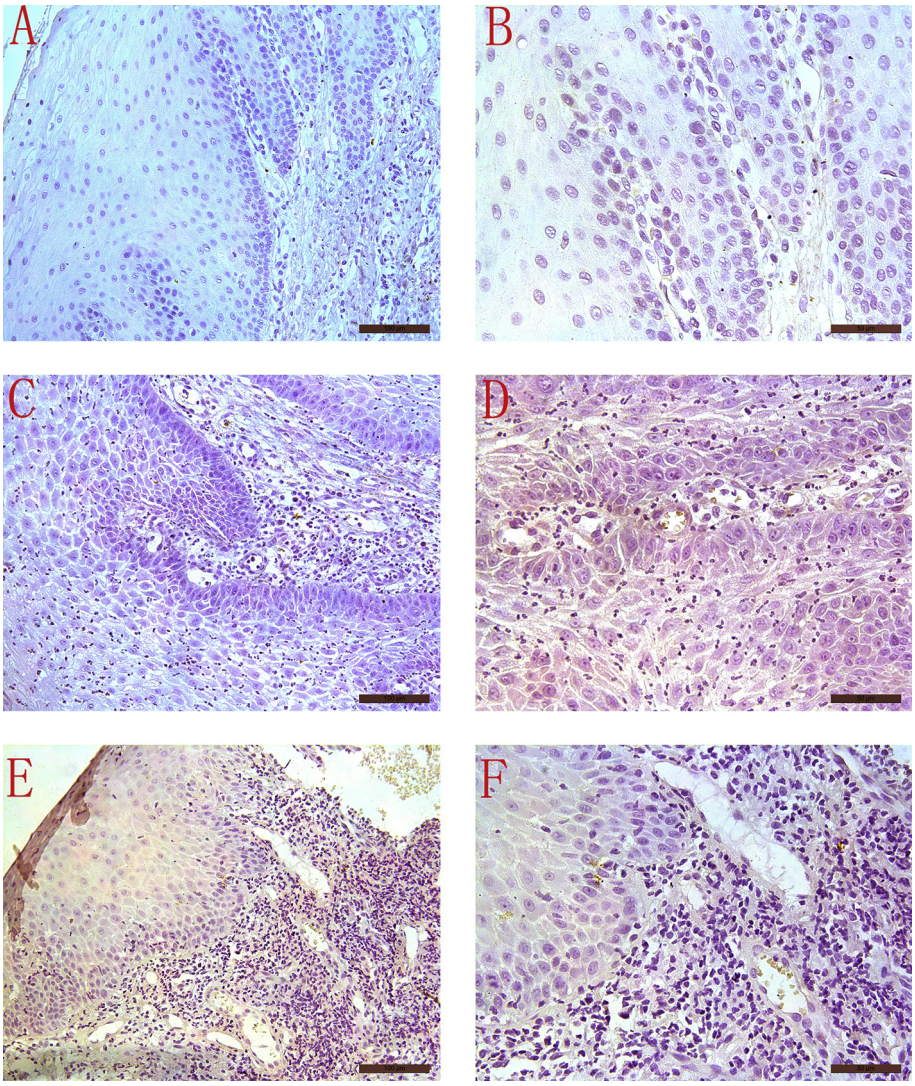

Figure 9 No cells positive for hypoxia-inducible factor/vascular endothelial growth factor-C positive cell were found in negative controls. (A, B) Healthy control group. (C, D) Moderate periodontitis group. (E, F) Advanced periodontitis group. Original magnification: A, C, and E, 200×; B, D, and F, 400×.

The binding of HIF-1α to the HRE in the VEGF promoter is a predominant inducer of VEGF expression.[38] The role of VEGF-C in inflammation is not fully understood. In chronic airway inflammation induced by *Mycoplasma pulmonis* infection or in chronic arthritis induced by the overexpression of TNF,[40] systemic inhibition of the VEGF receptor 3 signaling pathway caused exaggerated tissue ischemia, edema, and damage. However, there is no report that VEGF-C has immunomodulatory effects on periodontitis. In the present study, our results showed that VEGF-C was weakly expressed in healthy tissues, and there was significantly higher expression in connective tissue papilla of gingival tissues with chronic periodontitis; the expression level in advanced chronic periodontitis was higher than that of moderate chronic periodontitis. In our study, the distribution of VEGF-C protein was marked in connective tissue papilla of gingival tissues with chronic periodontitis, but there was significantly reduced staining of VEGF-C protein in the epithelial layer. The changes in the vascularity of the periodontal connective tissues in advanced periodontitis may be, in part, a consequence of altered expression of angiogenic activity by the epithelium. In turn, this may reflect the epithelial response to microbial flora in the microenvironment of the periodontal pocket.[41] The Pearson correlation analysis showed a positive correlation between the expression level of HIF-1α and VEGF-C in gingival tissues with periodontitis. These results may be explained by the fact that the signal of VEGF-C is upregulated by HIF-1α in the pathogenesis of periodontitis. The expression of HIF-1α correlates with hypoxia-induced angiogenesis as a result of the induction of the major HIF-1 target gene VEGF.[42] Many studies have focused on the signaling pathway leading the regulatory processes of the HIF-1 transcription factor.[43] Our findings suggested that VEGF-C protein expression may be regulated by HIF-1α, involving the inflammation and regulation of immune responses in the pathogenesis of periodontitis. The expression level of HIF-1α and VEGF-C in chronic periodontitis was higher than that in healthy

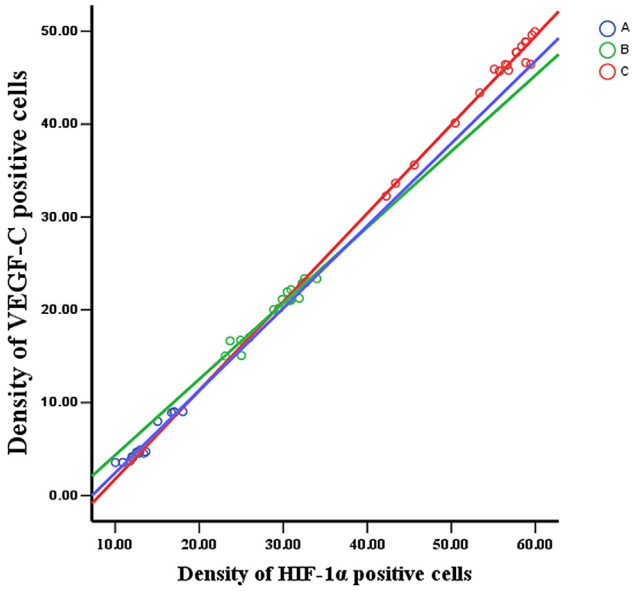

Figure 10 Correlation analysis between density of HIF-1α-positive cells and VEGF-C-positive cells. (A) Healthy control group ($n = 16$). (B) Moderate periodontitis group ($n = 20$). (C) Advanced periodontitis group ($n = 20$). Pearson correlation analysis showed that there was a positive correlation between expression level of HIF-1α and VEGF-C in gingival tissues in the periodontitis groups and the healthy controls. There was a strong correlation between expression of HIF-1α, VEGF-C, and severity of human chronic periodontitis. HIF = hypoxia-inducible factor; VEGF = vascular endothelial growth factor.

controls. Meanwhile, a higher expression level of HIF-1α and VEGF-C were observed in advanced chronic periodontitis than that of moderate periodontitis. Similar results were found in oral squamous cell carcinoma.[44] Klatte et al[45] demonstrated that the high expression of HIF-1α was significantly associated with signals of the cell cycle, apoptosis and expression of some crucial proteins such as VEGF, platelet-derived growth factor, and epidermal growth factor receptor. Under normoxic conditions, HIF-1α expression is maintained at low steady-state levels by the critical oxygen sensor prolyl hydroxylase 2. In chronic periodontitis, HIF-1α is expressed highly in gingival fibroblasts and upregulates inflammatory factor transcription, which promotes periodontal inflammation.[46] In an *in vitro* study investigating the effects of hypoxia on apoptosis and autophagy of human periodontal ligament cells, it was demonstrated that hypoxia induces apoptosis and autophagic cell death in human periodontal ligament cells through the HIF-1α pathway.[47]

Our results showed that the HIF-1α protein is mainly expressed in the epithelial layer of gingival tissues, and VEGF-C protein is mostly located in connective tissue papilla of gingival tissues. Our study indicates that hypoxia may play an important role in the pathogenesis of periodontitis. Our first focus shows that there exists a strong correlation between the expression of HIF-1α and VEGF-C and severity of human chronic periodontitis. The results suggest that HIF-1α may affect VEGF-C expression, thus acting as a crucial regulator in the pathogenesis of periodontitis. This study highlights the potential of HIF-1α as a therapeutic target against periodontitis.

Conflicts of interest

All authors declare no conflicts of interest.

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