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Up-regulation of vascular endothelial growth factor in synovial fibroblasts from human temporomandibular joint by hypoxia

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INTRODUCTION: Enhanced expression of vascular endothelial growth factor (VEGF) has been described in patients with internal derangement (ID). Herein, we examined the expression of VEGF in synovial fibroblasts from temporomandibular joint (TMJ) under hypoxia and investigated the regulation of hypoxia-inducible factor-1α (HIF-1α) involved in the expression of VEGF.

METHODS: Synovial fibroblasts were prepared from human TMJ. These cells were incubated under hypoxia or normoxia for the indicated time periods. VEGF levels in cultured supernatant were measured by an ELISA. VEGF mRNA isoforms and stability were assessed using RT-PCR and Northern blot analysis respectively. HIF-1α accumulation was evaluated by Western blotting and immunofluorescence.

RESULTS: VEGF were significantly induced by hypoxia in synovial fibroblasts. In response to hypoxia, VEGF121 and VEGF165 mRNA were both remarkably increased, while there was no change in VEGF mRNA stability. The accumulation and nuclear translocation of HIF-1α occurred under hypoxia.

CONCLUSIONS: Hypoxia may mainly induce the expression of VEGF121 and VEGF165 in synovial fibroblasts to promote inflamed angiogenesis of TMJ. HIF-1α, which is clearly activated in response to hypoxia, may control the expression of VEGF in synovial fibroblasts from TMJ.

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Keywords: hypoxia; vascular endothelial growth factor; synovial fibroblasts; temporomandibular joint disorders; synovitis

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Introduction

The synovial membrane of temporomandibular joint (TMJ) is believed to exert a pivotal function in maintaining normal joint physiology and in joint pathology (1). The arthroscopic and histopathological studies on painful TMJ disorders have demonstrated the occurrence of inflammatory reactions in the synovial membrane, which is characterized by synovial lining cell layer hyperplasia, angiogenesis and inflammatory cell infiltration (2). The activated cells in the TMJ synovial microenviroment, particularly fibroblasts, produce cytokines to promote angiogenesis (3) and extracellular matrix degradation (4), as well as chemokines to recruit immunocytes (5).

Vascular endothelial growth factor (VEGF), which is a selective endothelial mitogen and vascular permeability factor, plays crucial roles in both normal and pathologic angiogenesis, such as embryonic development and ascites tumor formation (6). Enhanced expression of VEGF has been described in patients with internal derangement (ID) and it may be the key regulator for angiogenesis in synovium, disc, and fibrocartilage during the inflammatory process of TMJ (3, 7). Recent reports have demonstrated that up-regulation of VEGF may be involved in angiogenesis of synovitis via paracrine pathway and erosion of the cartilage via autocrine pathway in arthritic TMJ (8). Moreover, the importance of the role of VEGF in the pathogenesis of inflammatory arthritis is emphasized by observations indicating that inhibition of VEGF is associated with a marked reduction in the inflammatory response in a model of collagen-induced arthritis (9). Collectively, increased the expression of VEGF is related to pathological process of TMJ disorders.

There is evidence suggesting that TMJ disorders accompanied with clenching, bruxism, or heightened muscular tension exhibits elevated intra-articular pressures that exceed the end-capillary perfusion pressures and create a hypoxic environment (10, 11). Hypoxia is

known to be a potent stimulus for the expression of VEGF. VEGF mRNA has been shown to be up-regulated by hypoxia in a variety of cell types and the increase of VEGF is thought to be a major mechanism for the hypoxia-induced angiogenesis (6). Hypoxic stimulation of VEGF, along with a number of other hypoxia-responsive genes, is mediated by a ubiquitous and highly conserved transcription factor, hypoxia-inducible factor-1 (HIF-1) (12). HIF-1 is a heterodimeric transcription factor composed of an oxygen-sensitive HIF-1α and a constitute HIF-1β subunit, which both belong to the family of basic helix–loop–helix and Per/Arnt/Sim (PAS) domain proteins (13). HIF-1 activity is primarily modulated by the abundance of HIF-1α. HIF-1α is rapidly degraded under normoxic conditions but is stabilized under hypoxic conditions, rapidly translocating to the nucleus, where it initiates the expression of VEGF (14).

Despite intensive studies, understanding of the effects of hypoxia on VEGF expression and HIF-1α regulation in the synovial fibroblasts from TMJ is still unknown. In this study, we examined the expression of VEGF in synovial fibroblasts from TMJ under hypoxia and investigated the regulation of HIF-1α involved in the expression of VEGF in these cells under hypoxia.

Materials and methods

Cell culture

Synovial membrane specimens were obtained from three patients (17, 28 and 60 years old) with condylar fracture undergoing arthrotomy treatment. The samples obtained from synovial regions without inflammation were verified based on a pathologic diagnosis. Informed consent was obtained, and the protocol was approved by the Human Research Ethics Committee, School & Hospital of Stomatology, Wuhan University. Synovial fibroblasts were prepared as described (15). Synovial fibroblasts were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2 in air.

Human periodontal ligament cells (HPLCs) were obtained from healthy periodontal tissue and grown in DMEM medium containing 10% FCS as previously described (16). HPLCs were morphologically similar to synovial fibroblasts, grew faster, and reached a higher cell density at confluence than did synovial fibroblasts.

Hypoxic conditions

Hypoxia was induced using an anaerobic Incubator Chamber (Yiwu freezing company, Yiwu, China). The chamber was deoxygenated by positive infusion of 5% CO_2 and 95% nitrogen gas mixture. Equal atmospheric pressure was ensured by a standardized pressure gauge affiliated with anaerobic Incubator Chamber. After 3–24 h, the medium was analyzed for percentage of oxygen using a blood gas analyzer (GMI incorporation, Ramsey, MN, USA). In addition, the blood gas analyzer was used to monitor ambient pH. For the normoxic conditions, cells were placed in the humidified incubator with atmosphere of 5% CO_2 and 95% air.

ELISA measurement of secreted VEGF

Synovial fibroblasts and HPLCs were plated at 5×10^4 cells per well in 24-well plates with 10% FCS medium. When the cells were grown to confluence, the medium was replaced with 8% FCS medium. After pre-incubated with 8% serum medium for 24 h, the cells were changed to a 1-ml fresh medium containing 4% FCS to start each experiment. After hypoxic culture for varying lengths of time, the medium was collected and stored at −80°C until assayed. An ELISA kit for human VEGF (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's instructions. The VEGF ELISA kit did not exhibit cross-reactivity with a series of soluble immunoactive molecules and the standard curve range was from 31.2 to 2000 pg/ml.

RT-PCR

During each culture period, total RNA was isolated from synovial fibroblasts using Trizol reagent (Gibco BRL) according to the manufacturer's instruction and quantitated by spectrophotometer (Shimadzu Corporation, Tokyo, Japan). First strand cDNA was synthesized from 1 μg of total RNA for 40 min at 48°C using the Superscript™ reverse transcription system (TaKaRa Biotechnology, Dalian, China) with oligdT primer. For amplification, the following sense and antisense primers for each molecule were used: VEGF (5′-CGAAAC CATGAACTTTCTGCTGTC-3′, 5′-TCACCGCCTC GGCTTGTCACAT-3′); Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) (5′-GAGGGGCCATCCAC AGTCTTCTG-3′, 5′-CCCTTCATTGACCTCAACTA CATGGT-3′). Amplification conditions were as follows: 30 cycles of 30 s denaturation at 94°C, 2 min extension at 72°C, 30 s annealing at 54°C for VEGF, or 30 s annealing at 58°C for GAPDH. PCR products were analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide. Images were scanned and the densitometric value of each band was analyzed using Photo Documentation and Imaging System and BIO-1D software (Vilber Lourmat Biotechnology, La Valle'e, Cedex, France).

Northern blot analysis

Total RNA (25 μg) was electrophoresed on 1% agarose gels in buffer containing 0.6% formaldehyde in 50 mmol/l HEPES (pH 7.8) and 1 mmol/l ethylenediamine tetraacetic acid. Gels were soaked in water to remove formaldehyde and RNA was electrotransferred onto nylon membranes and cross-linked by ultraviolet irradiation. Membranes were prehybridized for 1 h at 42°C and hybridized overnight at 68°C with a human VEGF cDNA probe. The human VEGF cDNA probe was prepared by performing RT-PCR with the primer for VEGF (5′-CGCGGATCCAGGAGTACCCTGAT GAG-3′, 5′-CCGGAATTCACATTTGTTGTGCTGT-3′). The 204-bp amplified fragment, which can recognize all VEGF transcripts (17), was subsequently subcloned into pGEM-T Easy Vector System (Promega, Madison,

WI, USA), labeled with ^{32}P using the Random Primers DNA Labeling System (Gibco BRL). Equal RNA loading was assessed with a probe against 18S RNA (Ambion, Austin, TX, USA).

Immunofluorescence for HIF-1α

Synovial fibroblasts were fixed with freshly prepared 4% formaldehyde in PBS for 10 min, washed with PBS. Following blockade of non-specific binding sites by incubation with 5% BSA in PBS for 30 min, the cells were incubated with a monoclonal anti-HIF-1α antibody (Santa Cruz Biotech, Santa Cruz, CA, USA). HIF-1α antibody-binding was detected by incubation with a Cy3-conjugated goat anti-mouse IgM (Boster, Wuhan, China) diluted at 1:500 with 5% PBS for 1 h at room temperature. The slides were washed five times with PBS, mounted and examined by fluorescence microscopy.

Western blotting analysis of HIF-1α

Synovial fibroblasts were washed twice in cold PBS, drained, and scraped from the T-75 plates with 150 μl of cold lysis buffer [150 mmol/l NaCl, 10 mmol/l Tris (pH 7.6), 1% Triton X-100, 20 mmol/l EDTA, 1 mmol/l PMSF, 5 μg/ml aprotinin] at 4°C for 30 min. Cell lysates were then centrifuged at 12 000 rpm at 4°C for 15 min. Supernatant was collected and protein concentration was measured with the Bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of cell lysates (60 μg) were electrophoresed in 8% or 10% sodium dodecyl sulfate polyacrylamide gels and were transferred to polyvinylidene difluoride membranes for 2 h at 4°C. The membranes were blocked with containing 5% BSA Tris-buffered saline (25 mmol/l Tris HCl, 0.2 mmol/l NaCl, 0.2% Tween 20, pH 7.6) for 1 h at room temperature. Subsequently, membranes were treated with the related primary antibodies at the following dilutions: monoclonal HIF-1α (1:500) and polyclonal actin (1:2000) (Santa Cruz Biotech, Santa Cruz, CA, USA). As a secondary antibody, horseradish peroxidase, conjugated to anti-rabbit IgG antibody was used. Bound antibodies were revealed with the enhanced chemiluminescence system (Amersham, Piscataway, NJ, USA).

Statistical analysis

Experiments were done in at least triplicate. Values of protein quantification for VEGF are expressed as mean ± SD. Difference was determined by student's *t*-test or one-way ANOVA, and $P < 0.05$ was considered statistically significant.

Results

Induction of VEGF protein by hypoxia in synovial fibroblasts from TMJ

Production of VEGF in synovial fibroblasts was examined in response to hypoxia. Fig. 1 showed the time course of VEGF levels from cells cultured with hypoxic or normoxic condition for 0, 8, 16, and 32 h. Hypoxia significantly increased VEGF levels at most of the time points assayed. After 32 h hypoxia, up-regulation of VEGF level was more than fivefold compared with normoxia.

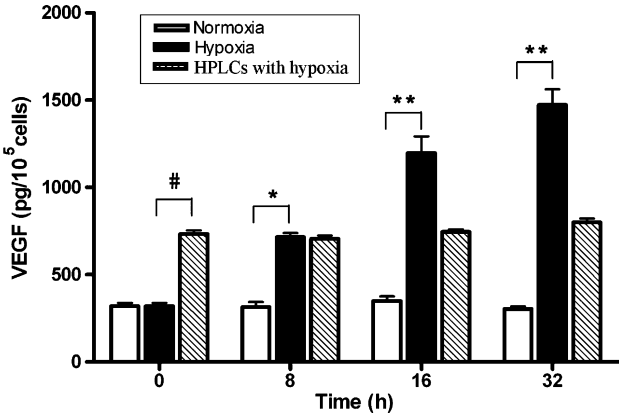

Figure 1 Time course of vascular endothelial growth factor (VEGF) release from synovial fibroblasts by hypoxia. VEGF protein levels in the media from synovial fibroblasts and periodontal ligament cell (HPLCs) cultured with hypoxic or normoxic condition for indicated time periods were determined. Results were expressed as mean ± SD ($n = 5$) of VEGF levels as pg/10^5 cells. $^*P < 0.05$, $^{**}P < 0.001$ (synovial fibroblasts under hypoxia vs. these cells under normoxia at corresponding time, *t* test); $^{\#}P < 0.05$ (synovial fibroblasts vs. HPLCs at 0 h, *t*-test), and there was no significant difference in VEGF levels for HPLCs at indicated times under hypoxia (one-way ANOVA).

In addition, to investigate whether hypoxia is a potent stimulus for VEGF production in synovial fibroblasts, VEGF levels from HPLCs were measured in identical hypoxic conditions. The baseline production of VEGF in HPLC was higher than it in synovial fibroblasts. After 32 h of hypoxia, there was no significant alteration in VEGF levels from HPLCs, whereas VEGF levels in the synovial fibroblasts reached as high as 1479.2 ± 163.7 pg/10^5 cells (Fig. 1).

Induction of mRNA for VEGF isoforms by hypoxia

To examine whether there was selectivity in the VEGF isoforms induced by hypoxic conditions, we performed RT-PCR on total RNA isolated from synovial fibroblasts using specific primers, which span exon 1 and 8 of VEGF gene (18). The expected sizes of the PCR products were 452, 584, 656, and 707 bp for VEGF121, VEGF165, VEGF189, and VEGF206 respectively. As shown in Fig. 2A, VEGF121 and VEGF165 were the dominant forms expressed in synovial fibroblasts in response to hypoxia. Furthermore, densitometric analysis of the bands for the VEGF121 isoform revealed that the expression of VEGF121 was up-regulated by hypoxia at all time points, with a maximum of more than 5.5-fold observed at 4 h (Fig. 2B).

VEGF mRNA stability under hypoxia

In addition, we used Northern blot analysis to investigate the mechanisms of VEGF mRNA up-regulation in synovial fibroblasts under hypoxia. Cells were pre-incubated in the hypoxic condition to allow synthesis of VEGF mRNA. After 4 h, 5 μg/ml of actinomycin D (Sigma, St Louis, MO, USA) was added to inhibit further transcription, and the level of VEGF mRNA was detected for the following 4 h. VEGF mRNA levels

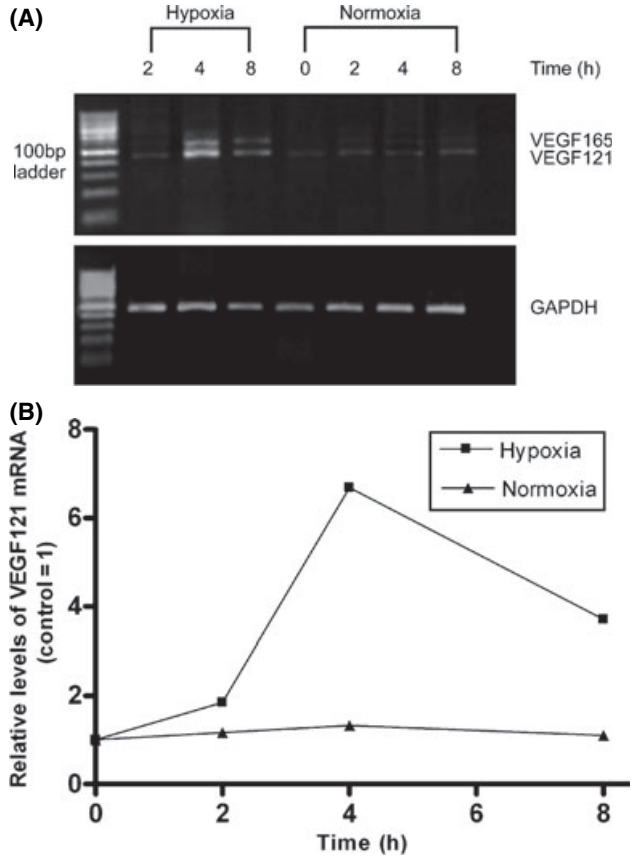

Figure 2 Induction of mRNA for vascular endothelial growth factor (VEGF) isoforms in synovial fibroblasts by hypoxia. (A) Synovial fibroblasts were incubated under hypoxia or normoxia for the indicated time periods. VEGF121 and VEGF165 were the dominant forms expressed in synovial fibroblasts in response to hypoxia. (B) VEGF121 expression was up-regulated by hypoxia at all time points, with a maximum of more than 5.5-fold observed at 4 h. The densitometric value of each band was nomalized by the ratio of GAPDH.

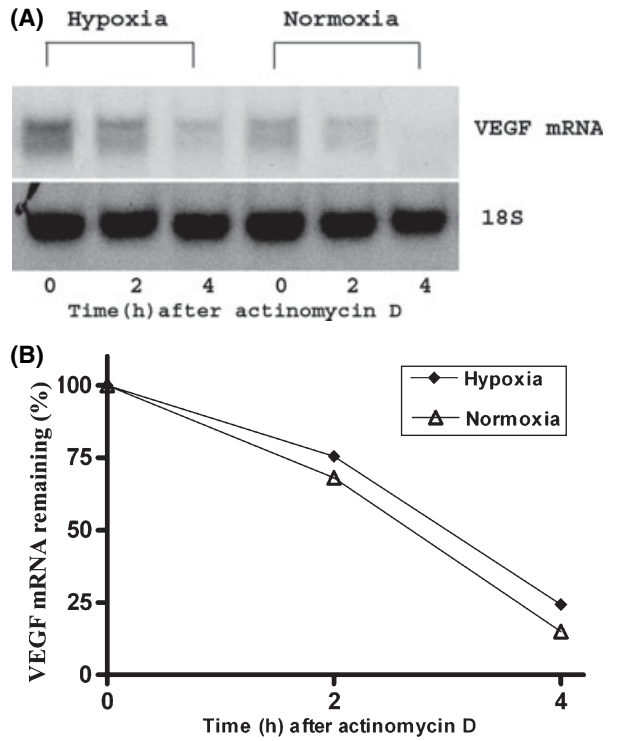

Figure 3 Effect of hypoxia on vascular endothelial growth factor (VEGF) mRNA stability in synovial fibroblasts. Cells were pre-incubated in the hypoxic condition to allow synthesis of VEGF mRNA. After 4 h, actinomycin D (5 μg/ml), was added to inhibit further transcription, and the level of VEGF mRNA was detected for the following 4 h by Northern blot analysis. In the graphic description of densitometry results, VEGF mRNA was normalized against 18S RNA, and the degradation rate was shown at 2–4 h and expressed as a percentage of the levels at 0 h.

decreased in hypoxia by approximately 80% within 4 h. A similar rate of degradation occurred in normoxia following addition of actinomycin D (Fig. 3).

Induction of HIF-1α accumulation and nuclear translocation by hypoxia

Because HIF-1 is the key regulator of hypoxia-induced gene expression, we analyzed whether synovial fibroblasts were able to accumulate the oxygen-sensitive subunit HIF-1α during hypoxia. Cells were exposed to hyoxia for 4 and 8 h. Significant HIF-1α accumulation was first detected after 4 h of hypoxia incubation by Western blotting analysis (Fig. 4A).

When immunofluorescence techniques were applied, the nuclear translocation of HIF-1α induced by hypoxia could be visualized in synovial fibroblasts over 8 h of hypoxia, while there was no staining under normoxic condition (Fig. 4B).

Discussion

Milam *et al.* (19) speculated that hypoxia-reperfusion injury may be an important model to elucidate the pathogenesis of TMJ inflammation. However, it is still unclear about the specific responses in TMJ experiencing hypoxia, which can result from tissue swelling and effusion in inflamed joints, and mechanical overload as well. Recently, some reports shed light on the effects of hypoxia on the TMJ disease. Hypoxia has been shown to modulate gene expressions of matrix metalloproteinases (MMPs) in TMJ disc cells, suggesting that a hypoxic environment may contribute to the pathogenesis of TMJ disorders (20). A hypoxia reperfusion model is used to explain the generation of free radicals in vascularized tissues of the TMJ (21).

Recent evidence indicates that synovial membrane is an active participant in the progression of TMJ disorders, rather than a passive target. Synovial fibroblasts from human TMJ in culture have been demonstrated to produce IL-6 (22), MMPs (4), and chemokines (5), such as IL-8, monocyte chemoattractant protein-1 (MCP-1), and RANTES, on stimulation with proinfammatory cytokines. Immunohistochemical studies indicate that synovial fibroblasts may also produce VEGF protein in the ID patients (3). It has been demonstrated that hypoxia is one of the most important factors to mediate VEGF production (6). In tissues with hypoxia, such as the ischemic hearts, the expression of VEGF provides a physiological feedback mechanism to overcome insufficient tissue oxygenation by promoting collateral vessel

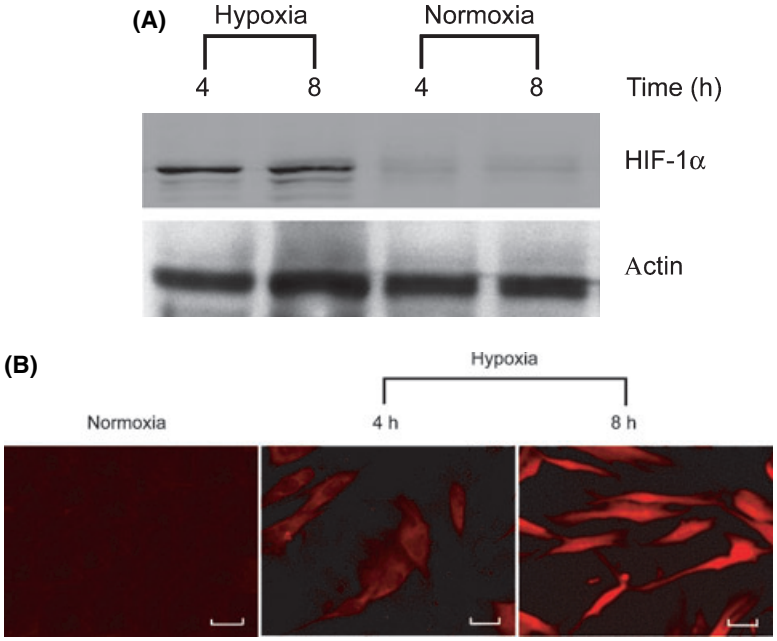

Figure 4 Induction of accumulation and translocation of HIF-1α in synovial fibroblasts by hypoxia (A) Western blot analysis for HIF-1α accumulation in synovial fibroblasts incubated under hypoxia or normoxia for 4 and 8 h. Actin was used to show equal loading. (B) Immunofluorescence staining of HIF-1α nuclear translocation in synovial fibroblasts over 8 h. Bar = 10 μm.

formation (23). *In vitro*, human synovial fibroblasts and endothelial cells from rheumatoid arthritis (RA) joints release VEGF in response to hypoxia (24, 25). In the present study, gene expression and protein production of VEGF can be induced by hypoxia in synovial fibroblasts of TMJ. To our knowledge, this is the first report to show that synovial fibroblasts from TMJ produce VEGF in culture.

In addition, recent studies have demonstrated that HPLCs have potential capacity to secrete VEGF (26). We also found that the levels of VEGF produced by HPLCs were more than twofold when compared with the levels by synovial fibroblasts in the normoxic condition. In the hypoxic conditions, however, VEGF levels in the synovial fibroblasts reached as high as 1479.2 ± 163.7 pg/10^5 cells after 32 h, while there was no significant alteration in VEGF levels from HPLCs. This comparison suggests that synovial fibroblasts from TMJ seem to have a remarkable potential to secrete VEGF under hypoxic condition.

The expression of VEGF exists as several isoforms that are produced by alternative splicing of the primary transcript (6). The bioactivities of VEGF differ among the isoforms. VEGF121 is closely correlated with vascular permeability and VEGF165 is a strongly potent endothelial cell mitogen, while the role of others is still poorly understood (27). It is known that metabolic stress conditions, such as hypoxia and glucose starvation, can differentially affect VEGF isoform mRNA expression (28). Birot et al. (29) reported that VEGF188 was increased in the heart in rats under hypoxia, whereas no change was found in VEGF 164 expression levels (VEGF isoforms are one amino acid shorter in mice). In RA joints, VEGF121 seems to be the dominant isoform under hypoxia in synovial fibroblasts (24). In contrast, our RT-PCR data showed that the expressions of VEGF121 and VEGF165 was both remarkably increased under hypoxia. Therefore, we suggest that for TMJ, hypoxia may mainly induce the expression of VEGF121 and VEGF165 in synovial fibroblasts to promote inflamed angiogenesis.

Numerous studies have reported that hypoxia induces VEGF gene expression by increasing VEGF mRNA in both transcriptional and post-transcriptional mechanism. Levy *et al.* (30) demonstrated that hypoxia prolonged VEGF mRNA half-time in PC12 rat pheochromocytoma cells using actinomycin D experiments. However, our results showed that VEGF mRNA of synovial fibroblasts presented a similar rate of degradation in hypoxic and normoxic conditions. Our data agree with work by Steinbrech *et al.* (31), who reported that hypoxic conditions did not cause significant alterations in VEGF mRNA stability in MC3T3-E1 cells. Thus, we suggest that for synovial fibroblasts in TMJ, hypoxia-induced VEGF gene expression is not related with the VEGF mRNA stability.

As post-transcriptional mechanism is not involved in VEGF induction by hypoxia for synovial fibroblasts, we examined whether HIF-1α, the pO_2-sensitive transcription factor, modulated VEGF gene. It has previously been reported that hypoxic induction of VEGF is mediated by HIF-1α, and a hypoxia-responsive element characterizing the known HIF-1α consensus-binding motif has been clarified in the VEGF promoter (32). Indeed, in the hypoxic synovium of RA, high expressions of HIF-1α and of VEGF were found by immunohistochemistry. In that study, a significant correlation between HIF-1α, VEGF expression, and degree of inflammation of joints was reported (33). However, there is no report on the expression of HIF-1α in TMJ

and it is not clear whether hypoxia stimulates up-regulation of VEGF expression in synovial fibroblasts through the classic pathway: HIF-1α activation. In this study, the accumulation of HIF-1α protein was observed in the synovial fibroblasts of TMJ under hypoxia. Moreover, the immunofluorescence staining showed that nuclear translocation of HIF-1α occurs in these cells over 8 h of hypoxia. The accumulation and translocation of HIF-1α induced by hypoxia indicates activation of HIF-1α. Therefore, these findings suggest that HIF-1α may control the expression of VEGF in synovial fibroblasts from TMJ under hypoxia.

In conclusion, our study describes the induction of VEGF121 and VEGF165 expression in TMJ synovial fibroblasts by hypoxia. HIF-1α, which is clearly activated in response to hypoxia, may control the expression of VEGF in synovial fibroblasts from TMJ. These results may provide new insights into the hypoxia-reperfusion injury mechanism involved in the pathogenesis of TMJ disorders.

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