

Role of hypoxia-inducible factor-1 α in hypoxia-induced expressions of IL-8, MMP-1 and MMP-3 in rheumatoid fibroblast-like synoviocytes

J. K. Ahn¹, E.-M. Koh¹, H.-S. Cha¹, Y. S. Lee², J. Kim³, E.-K. Bae³ and K.-S. Ahn³

Objectives. Hypoxia-inducible factor-1 α (HIF-1 α) is a master regulator in the cellular response to hypoxic conditions, and rheumatoid synovial tissue is known to exist under hypoxic conditions. Therefore, this study was conducted to determine the contribution of HIF-1 α to hypoxia-induced MMP and cytokine production in fibroblast-like synoviocytes (FLS).

Methods. RA FLS were transfected with either a plasmid that expresses HIF-1 α or an empty vector as a control, and then cultured under normoxia (21% O₂). Also, FLS were transfected with either HIF-1 α small interfering RNA (siRNA) or control siRNA, and cultured under hypoxic conditions (1% O₂). Following transfection, the amounts of MMP and cytokine mRNAs and HIF-1 α protein were examined using real-time RT-PCR and western blotting, respectively.

Results. The expression of HIF-1 α , MMP-1, MMP-3, IL-6 and IL-8 was markedly enhanced in FLS that were cultured under hypoxia. We confirmed that transient transfection of HIF-1 α overexpressing vector or siRNA had occurred using western blotting, and *in vitro* studies conducted using FLS transfected with HIF-1 α overexpression vector showed that they had significantly increased MMP-1, MMP-3 and IL-8 expression levels. Further, hypoxia-induced MMP-3 expression was significantly attenuated by knock-down of HIF-1 α , whereas hypoxia-induced IL-8 or MMP-1 expression was not significantly repressed by HIF-1 α siRNA.

Conclusions. Hypoxia-induced MMP-3 expression is exclusively regulated by HIF-1 α , and hypoxia-induced MMP-1 or IL-8 expression appears to have salvage pathways other than the HIF-1 α pathway. Together, these data provide new insight regarding the mechanism by which hypoxia participates in joint inflammation and destruction in RA.

KEY WORDS: Rheumatoid arthritis, Fibroblast-like synoviocytes, Hypoxia, Hypoxia-inducible factor-1 α , Matrix metalloproteinase, Cytokines.

Introduction

RA is a chronic inflammatory disease characterized by synovial inflammation and destruction of bone and cartilage that is mediated by pronounced tumour-like expansion of synovial fibroblasts, known as pannus [1]. Metabolically active synovium may require increased oxygen consumption and chronically inflamed joints generate high IA pressure due to the large amount of joint effusion and subsequent decreased blood supply. Therefore, it has been suggested that the rheumatoid synovial microenvironments exist under hypoxic conditions [2–4]. Previous studies have shown that rheumatoid SFs are hypoxic, acidic and exhibit low glucose and elevated lactate concentrations, which indicates that a shift to anabolic metabolism occurs in hypoxic synovium [5, 6]. Hypoxia-inducible factor-1 α (HIF-1 α), which is a key transcription factor in the cellular response to hypoxic conditions, is involved in angiogenesis, glycolysis, control of vascular tone and erythropoiesis [7]. Up-regulation of HIF-1 α occurs in cancer cells subjected to hypoxia [8, 9], and a hypoxia/HIF-1 α cascade in solid cancer is known to be associated with resistance to therapy and poor prognosis [7, 10]. HIF-1 α expression is also up-regulated in rheumatoid synovium [11, 12], and hypoxia/HIF-1 α is known to induce recruitment of inflammatory cells and angiogenesis in rheumatoid synovium [13–15]. In addition, it was recently reported that a marked attenuation of synovitis and pannus formation occurred in mice with a specific deletion of HIF-1 α in their myeloid lineage cells [16]. For these

reasons, it was assumed that HIF-1 α plays a critical role in the pathogenesis of RA.

A number of studies have suggested that fibroblast-like synoviocytes (FLS) are active participants in the progression of RA [17]. However, although inflammatory cytokines and MMPs produced by FLS are abundant in SF [18], and the rheumatoid synovial microenvironments are hypoxic [2–4], few studies regarding the role of HIF-1 α in the production of these MMPs and cytokines in FLS have been conducted. Therefore, we conducted this study to determine if HIF-1 α modulates hypoxia-induced expression of MMPs and cytokines in cultured FLS. To accomplish this, we first determined whether hypoxia increased the expression of MMPs, cytokines and HIF-1 α in FLS, and then elucidated the contribution of HIF-1 α to hypoxia-induced expression of MMPs and cytokines by overexpression or knock-down of the HIF-1 α gene.

Materials and methods

Isolation and culture of rheumatoid FLS

The experimental protocols used in this study were approved by the local ethics committee and informed consent was obtained from each patient included in this study. Patients with RA were diagnosed according to the standards of the ACR [19]. Synovial tissue that was obtained from RA patients during total knee joint replacement surgery was minced, digested overnight with 5 mg/ml type IV collagenase (Sigma, Poole, UK) and 150 μ g/ml type I DNase (Sigma), and then separated from the undigested tissue by unit gravity sedimentation. After collecting the suspended cells into fresh tubes, the cells were harvested by centrifugation at 500 *g* for 10 min. The pellet was then washed twice with DMEM (Life Technologies, NY, USA) containing 10% fetal calf serum (FCS). Next, resuspended cells were plated at a concentration of 2 \times 10⁶ cells/ml in a total volume of 1 ml/200 mm² in T-25 culture flasks. Following overnight incubation, non-adherent cells were removed by replacing fresh culture medium, and the attached cells were cultured in DMEM containing 10% FCS, 50 U/ml penicillin,

¹Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, ²Department of Internal Medicine, Masan Samsung Medical Center, Masan and ³Center for Molecular Medicine, Samsung Biomedical Research Institute, Seoul, Republic of Korea.

Submitted 3 October 2007; revised version accepted 4 February 2008.

Correspondence to: E.-M. Koh, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon-Dong, Gangnam-Gu, Seoul 135-710, Republic of Korea. E-mail: emkoh@skku.edu

50 mg/ml streptomycin and 0.025 mg/ml amphotericin B until 90% confluent growth was observed. The primary cultured cells were then passaged three to five times over several weeks for subsequent experiments. The cultures comprised a homogeneous population of fibroblastic cells between the third and fourth passages.

Immunohistochemical staining

Rheumatoid synovium were obtained from RA joints following joint replacement surgery or synovectomy. Formalin-fixed, paraffin-embedded surgical specimens were sectioned at a thickness of 5 μ m, warmed to 60°C for 30 min, deparaffinized in xylene for 30 min and hydrated in graded series of alcohol. Antigen retrieval was performed by immersing in 10 mM citrate buffer and microwave oven for 20 min. The slides were incubated overnight at 4°C with mouse mAb against HIF-1 α (Abcam, MA, USA) in a 1:100 dilution. Negative controls were incubated in the same dilution of matched normal immunoglobulin G. After extensive washing with TBS for 20 min twice, the slides were incubated for 25 min with biotinylated anti-mouse secondary antibody (Dako, Ely, UK) at room temperature. Then, peroxidase blocking solution (Dako) was added for 20 min. After washing in TBS, a LSAB2 kit based on the streptavidin-biotin-peroxidase reaction was used. Peroxidase activity was then measured using a 3,3'-diaminobenzidine (DAB) substrate. All slides were counterstained with Harris haematoxylin (YD diagnostics, Seoul, Korea) and dehydrated in a graded series of alcohols and xylene, and coverslips were applied with permount mounting media (Fisher Chemicals, PA, USA) under standard conditions.

Hypoxic culture conditions

FLS were exposed to hypoxic conditions for each experiment by incubating them in an atmosphere composed of 1% O₂, 5% CO₂ and 94% N₂ that was created using a hypoxic chamber (ASTEC, Japan). The medium was changed to serum-free medium immediately before the FLS were subjected to the hypoxic conditions, and for each experiment, parallel cultures were grown in serum-free medium under normoxic conditions in a 5% CO₂ incubator (Sanyo Co., Osaka, Japan). After culturing the FLS under these conditions for 24 h, the cells were harvested for subsequent experiments. Cell viability was determined by trypan blue dye exclusion.

Transfection of HIF-1 α overexpression plasmid vector and small interference RNA

Transient transfection of FLS was performed using the Amaxa Human Dermal Fibroblast Nucleofector kit (NHDF-adult; Amaxa Biosystems, Gaithersburg, MD, USA), according to the manufacturer's protocols. Briefly, an expression plasmid containing the full length HIF-1 cDNA was constructed in the pCMV6 expression vector (Rockville, MD, USA) and small interference RNA (siRNA) for HIF-1 α was purchased from Dharmacon (Lafayette, CO, USA). Next, overexpression plasmid vectors (5 μ g) or siRNA (1 μ g) for HIF-1 α (GenBank accession number NM_001530) were added to 5×10^5 FLS suspended in 100 μ l of Nucleofector solution. The mixture was then transferred into the electroporation cuvette and subjected to electroporation using the U-23 program, according to the manufacturer's instructions. An empty pCMV6 plasmid vector or control siRNA (Dharmacon) was used as a negative control. Immediately after electroporation, the cells were suspended in 500 μ l of cell culture medium, and transferred into culture dishes or plates. The transfected cells were grown under hypoxic or normoxic conditions for 24 h, and any overexpression or knock-down effect of HIF-1 α was determined using western blot analyses.

Western blot analysis for HIF-1 α

Whole cell lysates (WCL) were prepared using RIPA buffer that contained 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% NP40, 0.1% SDS and 10 mM sodium deoxycholate. The concentration of WCL was determined using a Bio-RAD protein assay kit (Bio-Rad, Richmond, CA, USA). Next, 15 μ l of WCL was separated by electrophoresis on a 10% acrylamide gel using a constant voltage of 80 V for 2 h. The separated proteins were transferred electrophoretically onto nitrocellulose membranes that had been pre-blocked for 4 h at room temperature in TTBS [20 mM Tris-Cl, pH 8.0, 500 mM NaCl, 0.05% (v/v) Tween-20] containing 1% skimmed milk powder. The membranes were incubated overnight at 4°C with 1:1000-diluted monoclonal antibodies to HIF-1 α (mouse monoclonal anti-HIF-1 α antibody, Transduction Laboratories, KY, USA) in TTBS. The membranes were washed with TTBS three times for 15 min, and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG antibody (1:5000, Jackson ImmunoResearch, West Grove, PA, USA). Proteins were detected using an ECL western blot detection system (Amersham Life Sciences, Buckinghamshire, UK).

Real-time RT-PCR for production of MMPs and cytokines

Total RNAs were isolated from cultured FLS using Trizol reagent that contained phenol and guanidine isothionate in a monophasic solution following the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). A volume of 1 μ l of pooled RNA was reverse transcribed in a total reaction volume of 20 μ l containing 0.5 μ g random hexamer primer, 20 units RNasin[®] ribonuclease inhibitor and superscript[™] II reverse transcriptase (Invitrogen). The levels of MMPs, IL-6, IL-8 and β -actin mRNA expression were measured by real-time RT-PCR using an ABI PRISM 7000 sequence detector system (Applied Biosystems, Foster City, CA, USA). The real-time RT-PCR amplification was performed using the pre-developed Assay-on-demand gene expression set for IL-6 gene (Assay ID HS00174131_m1, GenBank accession number NM_000600, Applied Biosystems), IL-8 gene (Assay ID Hs00174103_m1, GenBank accession number NM_000584, Applied Biosystems), MMP-1 gene (Assay ID Hs00233958_m1, GenBank accession number NM_002421, Applied Biosystems), MMP-3 gene (Assay ID Hs00233962_m1, GenBank accession number NM_002422, Applied Biosystems) and human ACTB (β -actin) for the endogenous control (VIC/MGB Probe, Applied Biosystems) in combination with the TaqMan Universal PCR Master Mix. Quantitation of MMP-1, MMP-3, IL-6 and IL-8 mRNA expression was calculated using the absolute method provided by the manufacturer (Applied Biosystems). Analysis was performed using the ABI PRISM 7000 Sequence Detection software (Applied Biosystems). The expression level of selected genes in unknown samples was calculated as the ratio of the selected gene mRNA vs β -actin. Quantitation of the selected gene mRNA and β -actin were performed using a standard curve made from a known serial dilution of rheumatoid FLS. A negative control without template was included in each experiment.

Statistical analysis

All data are expressed as the mean \pm S.E.M. The statistical significance of the experimental results was determined using the Wilcoxon signed ranks test, and a *P*-value of <0.05 was considered statistically significant.

Results

Initially, we conducted an immunohistochemical analysis to detect the expression of HIF-1 α in RA synovial tissue. As shown in Fig. 1, the expression of HIF-1 α was observed in the lining layer of the rheumatoid synovium. We then attempted to determine if

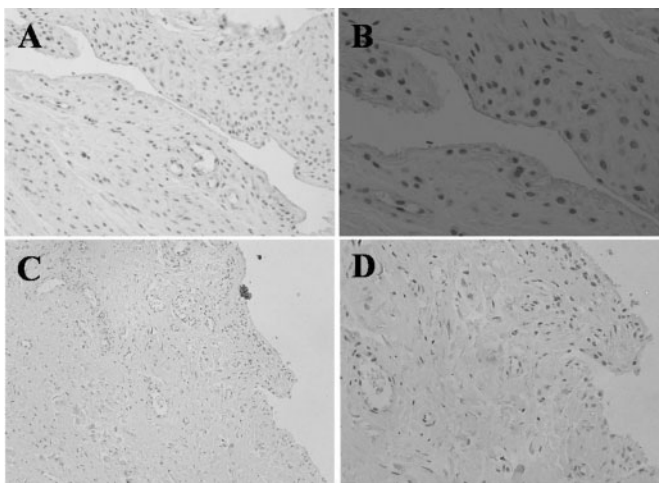


FIG. 1. Detection of HIF-1 α in synovial tissue of RA (A and B) and OA (C and D). Samples from rheumatoid synovium and osteoarthritic synovium were subjected to immunohistochemical staining for HIF-1 α . HIF-1 α staining was detected in some cells of rheumatoid synovium, whereas it had any detectable HIF-1 α expression in synovial tissue of OA (original magnification $\times 200$ in A and C; $\times 400$ in B and D).

hypoxia up-regulated the expression of HIF-1 α protein in RA FLS. To accomplish this, FLS obtained from five RA patients were cultured under normoxic or hypoxic conditions, and then examined for the presence of HIF-1 α protein using western blot analysis. The expression of HIF-1 α protein was found to be significantly higher in FLS that were cultured under hypoxic conditions than in FLS that were cultured under normoxic conditions (Fig. 2A), which is concordant with the results obtained when HIF-1 α expression in rheumatoid synovium was evaluated.

FLS obtained from six RA patients were cultured under normoxic or hypoxic conditions for 24 h. Quantitative determination of mRNA expression revealed that the levels of MMP-1, MMP-3, IL-6 and IL-8 were significantly increased in FLS under hypoxic conditions when compared with those under normoxic conditions ($P < 0.05$) (Fig. 2B), with the mean expression of MMPs and cytokines in our study increasing by ~ 2 - to 3-fold in FLS under hypoxic conditions when compared with those under normoxic conditions.

Next, to investigate the influence of HIF-1 α on the expression of MMPs, IL-6 and IL-8, FLS obtained from four RA patients were transfected with either HIF-1 α overexpression plasmid vectors or control empty vectors, and then cultured under normoxic conditions for 24 h. After confirmation of the transient

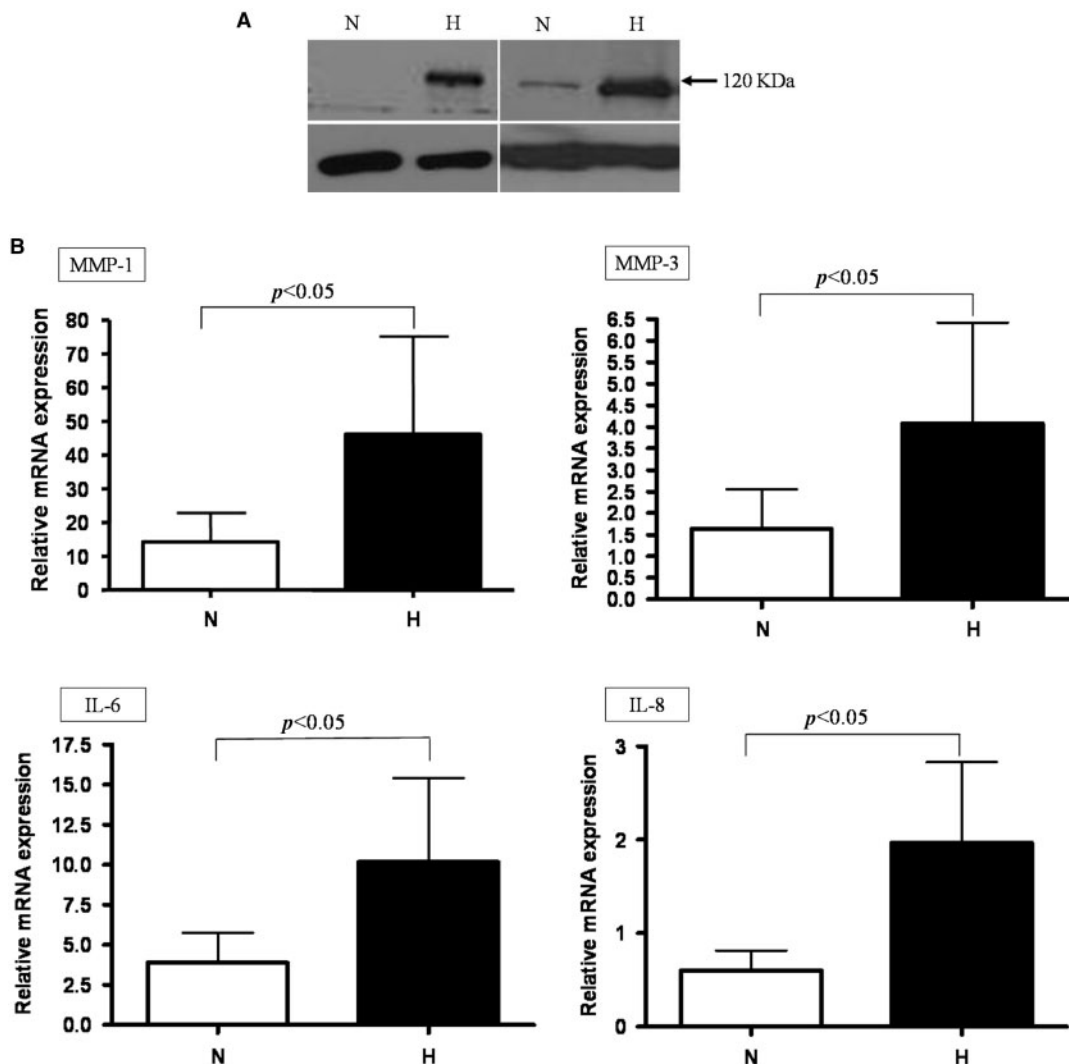


FIG. 2. Expression of MMPs, IL-6 and IL-8 mRNA and HIF-1 α protein in FLS cultured under normoxic or hypoxic conditions. Rheumatoid FLS were cultured under normoxic or hypoxic conditions for 24 h. The expressions of HIF-1 α protein were determined by western blotting. The levels of mRNA for MMPs, IL-6 and IL-8 were determined by real-time RT-PCR. Hypoxia increased the expression of HIF-1 α in rheumatoid FLS (A). The expressions of MMP-1, -3, IL-6 and -8 mRNA were significantly increased in FLS cultured under hypoxic conditions than in those cultured under normoxic conditions ($n = 6$) (B). Each real-time RT-PCR was performed in triplicate. N: FLS incubated under normoxic conditions; H: FLS incubated under hypoxic conditions.

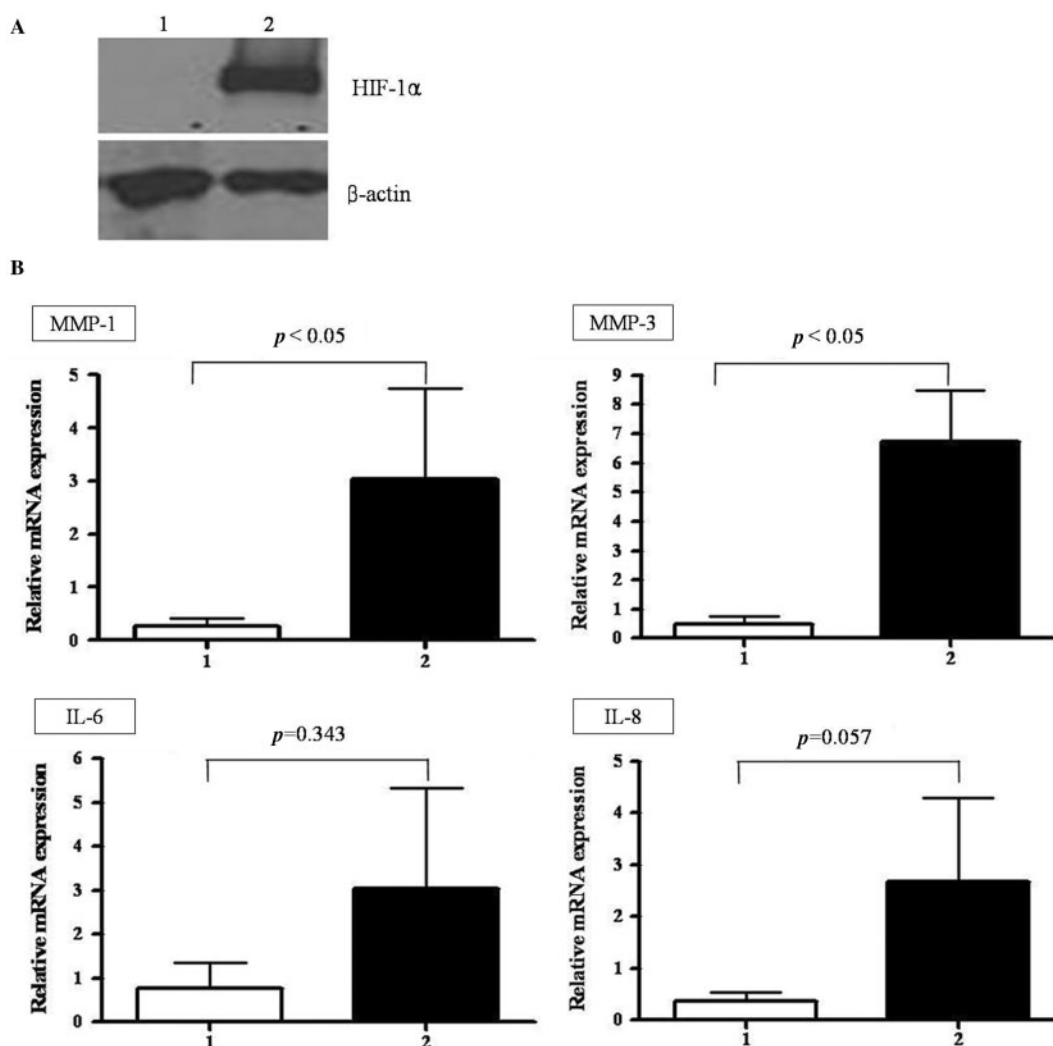


FIG. 3. Effect of HIF-1 α overexpression on the expression of MMPs, IL-6 and IL-8 in FLS. Primary FLS were cultured under normoxic conditions for 24 h after transient transfection of HIF-1 α overexpression vectors or control empty vectors. Western blot analysis showed that transient transfection of HIF-1 α overexpression vectors induced HIF-1 α protein expression in FLS (A). The mRNA expressions of MMPs, IL-6 and IL-8 were measured in FLS transfected with HIF-1 α overexpression vectors or control empty vectors by real-time RT-PCR ($n = 4$). MMP-1 and -3 expressions were significantly increased in FLS transfected with the HIF-1 α overexpression vectors (B). There was little difference in the expression of IL-6 observed in the HIF-1 α overexpression group and the control group. Each real-time RT-PCR was performed in triplicate. 1: control empty vector transfection; 2: HIF-1 α -overexpression plasmid vector 5 μ g transfection.

transfection of HIF-1 α overexpression plasmid vectors (Fig. 3A), the expression of MMPs, IL-6 and IL-8 was determined using real-time RT-PCR. As shown in Fig. 3B, the levels of MMP-1 and MMP-3 expression were greater in FLS that were transfected with the HIF-1 α overexpression plasmid than in those that were transfected with the control empty vectors ($P < 0.05$), with the mean level of MMP-1 and MMP-3 being increased by 11.6- and 14.1-fold, respectively, in HIF-1 α overexpression FLS when compared with control FLS. In addition, IL-8 expression tended to be greater in HIF-1 α overexpression FLS ($P = 0.057$) than in control FLS. However, transfection with HIF-1 α overexpression plasmid vectors had no effect on the expression of IL-6 ($P = 0.343$).

Next, we used HIF-1 α siRNA to analyse the contribution of HIF-1 α to hypoxia-induced MMP and cytokine expression in FLS. To accomplish this, we transfected FLS obtained from seven RA patients with either HIF-1 α or control siRNA, and then incubated the cells for 24 h under hypoxia. Western blot analysis revealed that the knock-down effect on HIF-1 α by HIF-1 α siRNA resulted in the complete suppression of hypoxia-induced HIF-1 α protein expression in FLS that were transfected with HIF-1 α siRNA (Fig. 4A), with MMP-3 mRNA expression in FLS that were transfected with HIF-1 α siRNA being reduced by $\sim 50\%$

when compared with FLS that were transfected with control siRNA ($P < 0.05$) (Fig. 4B). The expressions of hypoxia-induced MMP-1, IL-6 and IL-8 were not repressed by HIF-1 α siRNA.

Discussion

Rheumatoid synovitis is regulated by various cytokines, including IL-1, IL-6, IL-8 and TNF- α . The joint destruction is partially due to high activity of MMPs in the synovium [1]. Joints that are affected by RA are known to exist under hypoxic conditions and it is also known that hypoxia plays an important role in the pathophysiology of RA [20, 21]. However, the exact role that hypoxia plays in regulating MMPs and cytokines in the RA joint has yet to be determined [15, 22].

Our results demonstrated that hypoxia results in increased MMP-1, MMP-3, IL-6 and IL-8 expression. The results of the present study were consistent with previously published observations, showing that hypoxia is one of the stimuli for MMP-1, MMP-3, IL-6 and IL-8 expression [23–27]. These data suggest that hypoxia plays a pivotal role in the inflammation and subsequent joint destruction in RA through the induction of MMP and cytokine expression. However, little is known about the

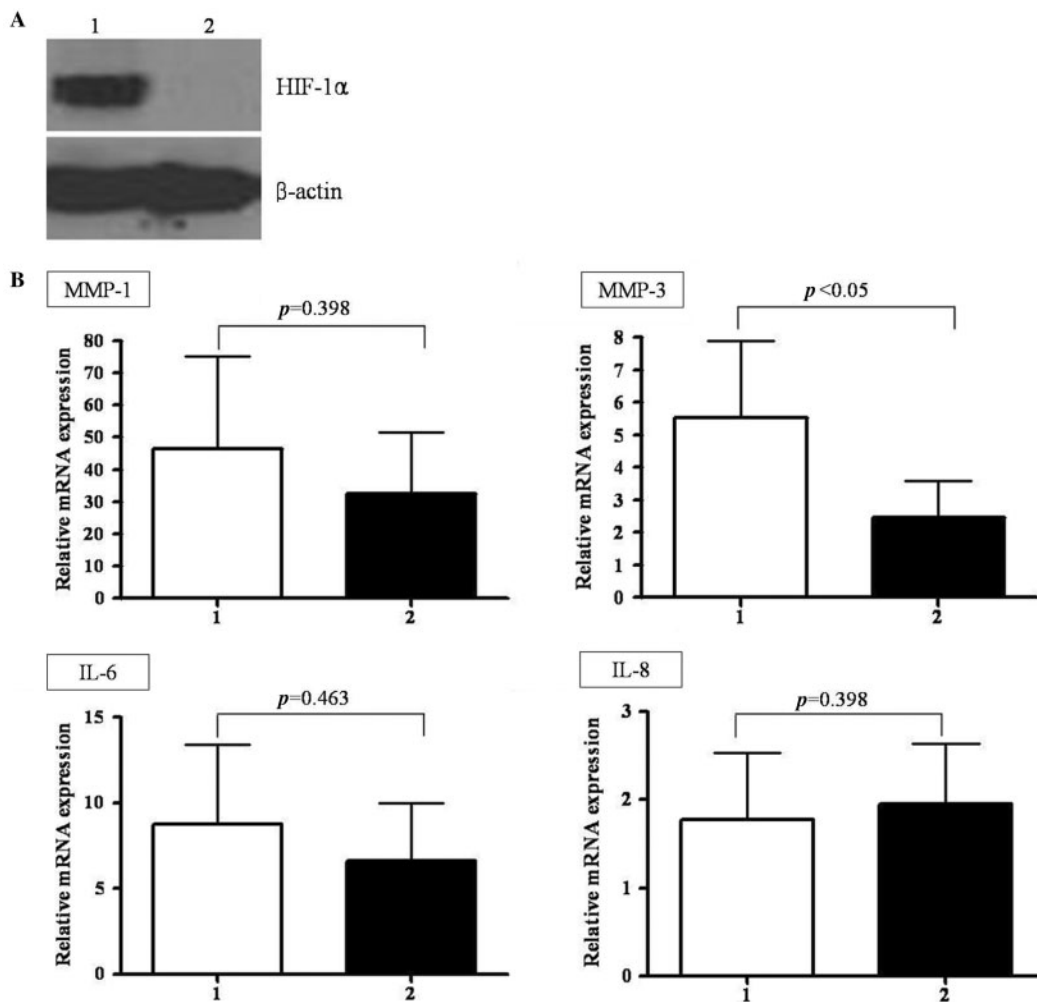


FIG. 4. Effect of HIF-1 α knock-down on the hypoxia-induced expression of MMPs, IL-6 and -8 in FLS. Rheumatoid FLS were transfected with HIF-1 α siRNA or control siRNA, and the levels of HIF-1 α proteins were then determined by western blot analysis 24 h after transient transfection. The levels of mRNA for MMPs, IL-6 and IL-8 were determined by real-time RT-PCR. HIF-1 α siRNA specifically reduced the HIF-1 α protein level in FLS (A). The levels of MMP, IL-6 and -8 mRNA expression were measured in FLS after transient transfection of HIF-1 α or control siRNA ($n=7$). Only MMP-3 expression was significantly decreased in FLS (B). Each real-time RT-PCR was performed in triplicate. 1: control siRNA; 2: HIF-1 α siRNA 1 μ g.

mechanism by which hypoxia induces the expression of MMPs and cytokines in FLS.

HIF-1 α may be one of the candidate transcription factors by which hypoxia exerts its effects because hypoxia is a potent stimulant of HIF-1 α [7] and HIF-1 α is expressed in rheumatoid synovial tissue and FLS [11, 12, 14]. HIF-1 α up-regulates enzymes involved in tumour invasion and metastasis, such as urokinase-type plasminogen activator, MMP-2 and cathepsin D [28], and there is a substantial body of evidence that suggests HIF-1 α might play a key role in synovial inflammation and joint destruction in RA [14–16, 29]. The deletion of myeloid HIF-1 α in K/BxN mice has been also shown to reduce joint swelling, synovial infiltration of macrophages and pannus formation [16]. Based on these observations, we hypothesized that HIF-1 α modulates the expression of MMPs and cytokines induced by hypoxia in cultured FLS. Thus, we conducted this study to test our hypothesis.

We initially demonstrated that the expression of HIF-1 α was enhanced in rheumatoid synovial tissue and FLS cultured in hypoxia. Considering controversy continues concerning the expression of HIF-1 α by rheumatoid FLS, these findings might provide additional information regarding the HIF-1 α expression. It is conceivable that the overexpression or knock-down of HIF-1 α influences the HIF-1 α binding activity, which would subsequently influence the expression of MMPs and cytokines.

Therefore, we determined the role of HIF-1 α on hypoxia-induced MMPs and cytokine expression by transfecting FLS with HIF-1 α overexpression plasmid vector and siRNA. We demonstrated that HIF-1 α overexpression caused significant up-regulation of MMP-3 in FLS. Moreover, significant suppression of hypoxia-induced MMP-3 expression occurred when HIF-1 α expression was suppressed by RNA interference. The expression level of MMP-3 was clearly paralleled by the changes in HIF-1 α expression, which indicates that HIF-1 α is involved in the direct regulation of hypoxia-induced MMP-3 expression. To our knowledge, this is the first report to show that hypoxia-induced MMP-3 expression is regulated by HIF-1 α transcription factor in FLS.

The levels of MMP-1 and IL-8 expression were considerably greater in FLS that had been transfected with HIF-1 α overexpression plasmid vectors, but hypoxia-induced MMP-1 and IL-8 expressions were not abrogated by HIF-1 α siRNA. These observations suggest that IL-8 and MMP-1 may be additionally controlled by transcription factors other than HIF-1 α . While it was recently reported that hypoxia-stimulated IL-8 expression is dependent on the activation of HIF-1 α in human endothelial cells [30], it has also been shown that NF- κ B transcription factor regulates IL-8 gene transcription in prostate cancer cell lines under hypoxic conditions [31]. These conflicting data may have been due to different types of cells and diseases being studied.

Because NF- κ B is a key transcription factor in response to hypoxia [32] and it increases IL-6 expression [18], it may stand to reason that the expression of IL-6 mRNA was not influenced by overexpression or knock-down of HIF-1 α in our study. HIF-1 α and NF- κ B are both thought to be regulated by oxygen concentrations and both play important roles in synovitis and joint destruction through increased expression of hypoxia-related genes. Therefore, the interaction between HIF-1 α and NF- κ B under hypoxic conditions needs to be elucidated.

A limitation of this study is that double-labelling with FLS-marker was not performed in immunohistochemical staining for HIF-1 α to confirm that FLS express HIF-1 α . However, as shown in Fig. 2A, the western blot analysis for HIF-1 α in FLS is thought to be enough to confirm that FLS under hypoxia express HIF-1 α proteins. One potential problem is that there is no established cell line in the research for FLS, and this may explain the large standard error in our study.

In conclusion, the expression of MMP-1 and IL-8 appears to be partially regulated by HIF-1 α under hypoxic conditions. A key finding in this study is that hypoxia-induced MMP-3 expression is exclusively regulated by HIF-1 α . These results provide us with new insight regarding the mechanism by which the microenvironmental influence on synovial inflammation occurs, and will have implications in the development of more targeted therapy for RA.

Rheumatology key message

- Hypoxia-induced expression of MMP-3 is directly controlled by activation of HIF-1 α in FLS. A hypoxic microenvironment may lead to synovial inflammation and joint destruction via an HIF-1 α -dependent pathway.

Acknowledgements

Funding: This work was supported by grants from the Korea Science and Engineering Foundation (R01-2004-000-11007-0).

Disclosure statement: The authors have declared no conflicts of interest.

References

- Muller-Ladner U, Gay S. MMPs and rheumatoid synovial fibroblasts: Siamese twins in joint destruction? *Ann Rheum Dis* 2002;61:957–9.
- Edmonds SE, Blake DR, Morris CJ, Winyard PG. An imaginative approach to synovitis—the role of hypoxic reperfusion damage in arthritis. *J Rheumatol Suppl* 1993;37:26–31.
- Edmonds SE, Ellis G, Gaffney K, Archer J, Blake DR. Hypoxia and the rheumatoid joint: immunological and therapeutic implications. *Scand J Rheumatol Suppl* 1995;101:163–8.
- Blake DR, Winyard PG, Marok R. The contribution of hypoxia-reperfusion injury to inflammatory synovitis: the influence of reactive oxygen intermediates on the transcriptional control of inflammation. *Ann NY Acad Sci* 1994;723:308–17.
- Lund-Olesen K. Oxygen tension in synovial fluids. *Arthritis Rheum* 1970;13:769–76.
- Treuhart PS, Lewis MR, McCarty DJ. Synovial fluid pH, lactate, oxygen and carbon dioxide partial pressure in various joint diseases: a rapid method for evaluating the structure and function of the rheumatoid hand. *Arthritis Rheum* 1971;14:475–84.
- Harris AL. Hypoxia—a key regulatory factor in tumour growth. *Nat Rev Cancer* 2002;2:38–47.
- Paul SA, Simons JW, Mabeesh NJ. HIF at the crossroads between ischemia and carcinogenesis. *J Cell Physiol* 2004;200:20–30.
- Talks KL, Turley H, Gatter KC *et al*. The expression and distribution of the hypoxia-inducible factors HIF-1 α and HIF-2 α in normal human tissues, cancers, and tumor-associated macrophages. *Am J Pathol* 2000;157:411–21.
- Ikeda E. Cellular response to tissue hypoxia and its involvement in disease progression. *Pathol Int* 2005;55:603–10.
- Hollander AP, Corke KP, Freemont AJ, Lewis CE. Expression of hypoxia-inducible factor 1 α by macrophages in the rheumatoid synovium: implications for targeting of therapeutic genes to the inflamed joint. *Arthritis Rheum* 2001;44:1540–4.
- Giatromanolaki A, Sivridis E, Maltezos E *et al*. Upregulated hypoxia inducible factor-1 α and -2 α pathway in rheumatoid arthritis and osteoarthritis. *Arthritis Res Ther* 2003;5:R193–201.
- Sandau KB, Fandrey J, Brune B. Accumulation of HIF-1 α under the influence of nitric oxide. *Blood* 2001;97:1009–15.
- Hitchon C, Wong K, Ma G, Reed J, Lyttle D, El-Gabalawy H. Hypoxia-induced production of stromal cell-derived factor 1 (CXCL12) and vascular endothelial growth factor by synovial fibroblasts. *Arthritis Rheum* 2002;46:2587–97.
- Gaber T, Dziurla R, Tripmacher R, Burmester GR, Buttgerit F. Hypoxia inducible factor (HIF) in rheumatology: low O₂! See what HIF can do! *Ann Rheum Dis* 2005;64:971–80.
- Cramer T, Yamanishi Y, Clausen BE *et al*. HIF-1 α is essential for myeloid cell-mediated inflammation. *Cell* 2003;112:645–57.
- Firestein GS. Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? *Arthritis Rheum* 1996;39:1781–90.
- Sweeney SE, Firestein GS. Rheumatoid arthritis: regulation of synovial inflammation. *Int J Biochem Cell Biol* 2004;36:372–8.
- Arnett FC, Edworthy SM, Bloch DA *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;31:315–24.
- Wallis WJ, Simkin PA, Nelp WB. Low synovial clearance of iodide provides evidence of hypoperfusion in chronic rheumatoid synovitis. *Arthritis Rheum* 1985;28:1096–104.
- James MJ, Cleland LG, Rofe AM. Determinants of synovial fluid lactate concentration. *J Rheumatol* 1992;19:1107–10.
- Fava RA, Olsen NJ, Spencer-Green G *et al*. Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. *J Exp Med* 1994;180:341–6.
- Cha HS, Ahn KS, Jeon CH, Kim J, Song YW, Koh EM. Influence of hypoxia on the expression of matrix metalloproteinase-1, -3 and tissue inhibitor of metalloproteinase-1 in rheumatoid synovial fibroblasts. *Clin Exp Rheumatol* 2003;21:593–8.
- Ahn JK, Jeon CH, Koh JH *et al*. Effect of hypoxia on cytokine production in rheumatoid fibroblast-like synoviocytes. *J Korean Rheum Assoc* 2003;10:30–8.
- Sack U, Kinne RW, Marx T, Heppt P, Bender S, Emmrich F. Interleukin-6 in synovial fluid is closely associated with chronic synovitis in rheumatoid arthritis. *Rheumatol Int* 1993;13:45–51.
- Cohen T, Nahari D, Cerem LW, Neufeld G, Levi BZ. Interleukin 6 induces the expression of vascular endothelial growth factor. *J Biol Chem* 1996;271:736–41.
- Xie K. Interleukin-8 and human cancer biology. *Cytokine Growth Factor Rev* 2001;12:375–91.
- Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003;3:721–32.
- Blades MC, Ingegnoli F, Wheller SK *et al*. Stromal cell-derived factor 1 (CXCL12) induces monocyte migration into human synovium transplanted onto SCID mice. *Arthritis Rheum* 2002;46:824–36.
- Kim KS, Rajagopal V, Gonsalves C, Johnson C, Kalra VK. A novel role of hypoxia-inducible factor in cobalt chloride- and hypoxia-mediated expression of IL-8 chemokine in human endothelial cells. *J Immunol* 2006;177:7211–24.
- Maxwell PJ, Gallagher R, Seaton A *et al*. HIF-1 and NF- κ B-mediated upregulation of CXCR1 and CXCR2 expression promotes cell survival in hypoxic prostate cancer cells. *Oncogene* 2007;26:7333–45.
- Walmsley SR, Print C, Farahi N *et al*. Hypoxia-induced neutrophil survival is mediated by HIF-1 α -dependent NF- κ B activity. *J Exp Med* 2005;201:105–15.