Hypoxia differentially affects IL-1β-stimulated MMP-1 and MMP-13 expression of fibroblast-like synoviocytes in an HIF-1α-dependent manner

Yeon-Ah Lee¹, Hyun M. Choi², Sang-Hoon Lee¹, Seung-Jae Hong¹, Hyung-In Yang¹, Myung C. Yoo² and Kyoung S. Kim²

Abstract

Objectives. To further understand the expression regulation of MMP-1 and MMP-13 under physiological and pathological conditions, we investigated the combined effects of hypoxia and pro-inflammatory stimuli on the expression of MMP-1 and MMP-13 in rheumatoid synovial fibroblasts.

Methods. Synovial fibroblasts were cultured under either hypoxic or normoxic conditions in the presence of IL-1β stimulation. The culture supernatant was analysed for secreted levels of VEGF, MMP-1 and MMP-13. Their gene expression was quantified with real-time and semi-quantitative PCR. Another group of cells was transfected with small-interfering RNA (siRNA) specific for hypoxia-inducible factor-1α (HIF-1α). The protein levels of HIF-1α were detected by western blot analysis.

Results. In response to 10 ng/ml of IL-1β under normoxia, the levels of MMP-1 and MMP-13 increased compared with the levels observed under hypoxia. IL-1β stimulation under hypoxia induced a 2-fold increase in the level of MMP-1 and a 2-fold decrease in the level of MMP-13 compared with cells cultured under normoxia. A similar pattern of differential expression for MMP-1 and MMP-13 was observed with 1 and 5 ng/ml IL-1β, but not at 0.1 ng/ml. The differential expression of MMPs under the combined effect of IL-1β and hypoxia was significantly attenuated by silencing HIF-1α with siRNA.

Conclusions. Hypoxia in arthritic joints may differentially affect the IL-1β-stimulated expression of MMP-1 and MMP-13 in rheumatoid synovial fibroblasts. This effect is dependent on HIF-1α expression. This hypoxia-mediated differential effect should be taken into consideration when testing the efficiency of therapies that target HIF-1α.

Key words: small-interfering RNA, hypoxia-inducible factor-1α, vascular endothelial growth factor, matrix metalloproteinase, fibroblast-like synoviocytes.

Introduction

Hypoxia is one of the primary characteristics of inflamed joints in RA [1]. Hypoxic conditions play an important role in rendering synovial fibroblasts pro-angiogenic and pro-invasive, thus, hypoxia leads to the debilitating features that are characteristic of RA [2]. The angiogenic and invasive behaviours of RA fibroblast-like synoviocytes (FLSs) appear to be dependent on the cooperative action of VEGF and MMPs, which are stimulated by hypoxia in synovial fibroblasts [3]. Thus, VEGF and MMPs have been recognized as therapeutic targets for treatments aimed at delaying RA symptoms in arthritis [4, 5]. In fact, in addition to hypoxia, pro-inflammatory cytokines may affect VEGF and MMP gene expression in synoviocytes during progressive inflammation. In particular, synovial fibroblasts produce large amounts of MMPs when they are stimulated by pro-inflammatory cytokines, including IL-1β and TNF-α, during joint inflammation in RA or OA [6]. The application of TNF-α-blocking agents significantly decreased the levels of MMP expression [7, 8]. Although both hypoxia and pro-inflammatory stimuli have been shown to
affect the expression of MMPs in inflamed joints in RA, most studies have focused on the effects of hypoxia alone, without considering the effects of inflammatory stimuli.

Among 20 different members of the MMP family [9, 10], MMP-1 and MMP-13 appear to be the most important in cartilage degradation in RA and OA because they are rate-limiting components of the collagen degradation process [11, 12]. MMP-1 and MMP-13 have the unique ability to cleave the native triple helix of collagen, thereby allowing the chains to unwind; the chains then become susceptible to further degradation by other MMPs. Thus, inhibitors with varying degrees of selectivity for different MMPs have shown cartilage protective activity in animal models of OA [13]. However, the development of MMP inhibitors as therapeutic agents has been hampered by side effects observed in clinical trials [14, 15]. Therefore, factors that affect individual MMP expression require further clarification to enable efficient control of MMP expression in clinical treatments.

Hypoxia-inducible factor-1α (HIF-1α) is involved in both angiogenesis and inflammation in RA due to its modulation of VEGF, MMPs and inflammatory cytokines and chemokines. Thus, HIF-1α perpetuates the destructive cascade of reactions. Moreover, IL-1α and TNF-α are relevant to RA and hypoxia because they modulate HIF levels [16]. Therefore, transcription factors have become a focus for therapeutic targets in RA [17]. This study aimed to gain insight into the regulation of MMP expression in arthritic joints. We investigated the combined effects of hypoxia and IL-1β on the expression of MMP-1 and MMP-13 in RA FLSs and determined whether the effect was modulated by HIF-1α.

Materials and methods

Cell culture and reagents

All in vitro experiments were carried out with FLSs derived from patients with RA. After obtaining informed consent according to the Declaration of Helsinki, synovial tissues were collected from RA patients. They met the 1987 ACR criteria for the diagnosis of RA and had been treated with non-biological DMARDs and were undergoing therapeutic joint surgery. FLSs were isolated as described previously [18] and grown in DMEM (low glucose) (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-BRL) and 1× antibiotic-antimycotic (Gibco-BRL). After the cells had grown to confluence, they were split at a 1:4 ratio. FLS passages 3–6 from patients were used for all experiments. FLSs were pre-incubated with serum-free media for 2 h and cultures were replaced with serum-free media before IL-1β (10 ng/ml for 24 h) purchased from R&D Systems, Inc. (Minneapolis, MN, USA) under hypoxia or normoxia (5% CO₂, 37°C). Hypoxic conditions were generated by incubating the cells at 2% O₂ in a hypoxic chamber gassed with a combination of N₂ and CO₂ (Invitrogen, 200, Ruskinn Technology Ltd, Pencoed, UK). This study was approved by the Institutional Review Board of Kyung Hee University Hospital at Kangdong.

Measurement of mRNA level by PCR

After the culture supernatants were harvested, the cells were used to measure gene expression levels. Trizol reagent (Invitrogen) was used to extract total RNA from the cells. Complementary DNA was synthesized from 1 μg of total RNA in 20 μl of reverse transcription reaction mixture containing 5 mM of MgCl₂, 1× reverse transcription buffer, 1 mM of dNTP, 1 U/μl of RNase inhibitor, 0.25 U/μl of avian myeloblastosis virus (AMV) reverse transcriptase and 2.5 μM of random 9-mers. For semi-quantitative PCR, aliquots of cDNA were amplified with the primers in a 25-μl PCR mixture containing 1× PCR buffer, 0.625 U of TaKaRa Ex Taq HS (TaKaRa Bio, Kyoto, Japan) and 0.2 μM of specific upstream primers, according to the protocol of the manufacturer. The PCR conditions for the VEGF and MMPs were as follows: 30-33 cycles of 95°C for 45 s, 55-60°C for 45 s and 72°C for 45 s. PCR products were subjected to electrophoresis in 1.5% agarose gels containing ethidium bromide, and the bands were visualized under UV light. For real-time quantitative PCR, the reaction was carried out using the LightCycler PCR system (Roche Diagnostics, Meylan, France), with the DNA-binding SYBR Green I dye used to detect the PCR products. A serial dilution was used to generate each standard curve. Each 20-μl reaction mixture contained 1× Light Cycler-DNA Master SYBR Green I dye used to detect the PCR products. A serial dilution was used to generate each standard curve.

Transfection of small interference RNA

FLSs were transiently transfected with siRNAs that targeted HIF-1α (Dharmacon, Lafayette, CO, USA) in Lipofectamine 2000 (GIBCO) according to the manufacturer’s protocols. Briefly, siRNA (1 μg) for HIF-1α (GenBank accession number NM_001530) was suspended in 100 μl of lipofectamine solution and mixed
with an equal volume of serum-free DMEM medium. The mixture was added to \(5 \times 10^5\) FLSs cultured in 100-mm dishes. Control siRNA (Dharmacon) was used as a negative control. After 6 h of incubation, the transfected cells were washed twice with PBS, replenished with fresh medium, and grown under hypoxic or normoxic conditions for 24 h. The knock-down of HIF-1\(\alpha\) was determined by western blot analysis. Three independent experiments were performed in quadruplicate. Each experiment was performed with synovial cells derived from different patients.

**Western blot analysis**

The transfected cells were subsequently washed twice in PBS and treated with 500 \(\mu\)l of lysis buffer [20 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 \(\mu\)g/ml chymostatin, 2 mM PMSF, 10 \(\mu\)M leupeptin and 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF)]. As described previously [18], the samples were separated using 12% SDS-PAGE, and were then transferred to Hybond-ECL membranes (Amersham, Arlington Heights, IL, USA). The membranes were first blocked with 6% non-fat milk dissolved in Tris buffered saline Tween2 (TBST) buffer [10 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.05% Tween-20]. The blots were then probed with various rabbit polyclonal antibodies for HIF-1\(\alpha\) and \(\beta\)-actin (Cell Signaling Technology, Beverly, MA, USA) diluted 1:1000 in Tris buffered saline (TBS) at 4°C for overnight, and incubated with 1:1000 dilutions of goat anti-rabbit IgG secondary antibody coupled with horseradish peroxidase. The blots were developed using the enhanced chemiluminescence (ECL) method (Amersham). For re-probing, the blots were incubated in the stripping buffer [100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7)] at 50°C for 30 min with occasional agitation.

**Statistical analysis**

All experiments were repeated three times, and the results are expressed as the mean (s.d.). Statistical evaluation was performed by means of the Mann–Whitney test. Differences were considered statistically significant at \(P < 0.05\).

## Results

**Effect of hypoxia on VEGF and MMP expression in synoviocytes stimulated with the pro-inflammatory cytokine, IL-1\(\beta\)**

We determined whether hypoxia could affect the expression of MMP-1 and MMP-13 in RA FLSs stimulated with pro-inflammatory cytokines. RA FLSs were stimulated with IL-1\(\beta\) under hypoxic or normoxic conditions. As shown in Fig. 1A, hypoxia alone did not significantly affect the level of MMP expression, but IL-1\(\beta\) stimulation under normoxia significantly increased the expression of MMP-1 and MMP-13 proteins. This increase in MMP-1 and MMP-13 expression was differentially affected by hypoxia. Under hypoxia, IL-1\(\beta\) mediated a 2-fold increase in MMP-1 and a 2-fold decrease in MMP-13 protein expression. Furthermore, the mRNA levels of these MMPs were similarly affected by hypoxia (Fig 1B and C).

To confirm that the combined treatment of IL-1\(\beta\) and hypoxia was not due to an alteration in the gene expression machinery in the cells, we tested the well-known effect of hypoxia on VEGF expression in this system. We found that VEGF expression in RA FLSs was significantly increased by hypoxia, both in the presence and absence of IL-1\(\beta\) stimulation. Hypoxia with IL-1\(\beta\) stimulation in RA FLSs showed a synergistic effect of about a 3-fold increase in VEGF protein expression compared with that observed in protein expression. This suggested that the system functioned appropriately in response to hypoxia.

IL-1\(\beta\) is a potent pro-inflammatory cytokine. For the above experiment, we used a concentration much higher than that found under physiological conditions. Therefore, we wondered whether the observed differential effect of hypoxia on the production of MMPs could be due to the non-physiological conditions of high IL-1\(\beta\) concentrations. Thus, we tested the effects of hypoxia on MMP expression in various concentrations of IL-1\(\beta\) (Fig. 2). We found that hypoxia induced a similar pattern of differential MMP-1 and MMP-13 expression with a weak 1 and 5ng/ml IL-1\(\beta\) stimulation, but not at 0.1 ng/ml IL-1\(\beta\). This suggested that a concentration of at least 1 ng/ml IL-1\(\beta\)

### Table 1 The sequence of PCR primers used in this experiment

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Product size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1 sense</td>
<td>5'-CCT AGC TAC ACC TTC AGT GG-3'</td>
<td>338</td>
</tr>
<tr>
<td>MMP-1 anti-sense</td>
<td>5'-GCC CAG TAC TTA CCT TT-3'</td>
<td>311</td>
</tr>
<tr>
<td>MMP-13 sense</td>
<td>5'-TGG AGG ATA CAG GCA AGA CT-3'</td>
<td>288</td>
</tr>
<tr>
<td>MMP-13 anti-sense</td>
<td>5'-ACT TCA GGC TCT CCT TT-3'</td>
<td>305</td>
</tr>
<tr>
<td>VEGF sense</td>
<td>5'-TTC AGA CAA CCT GAG TCC TT-3'</td>
<td></td>
</tr>
<tr>
<td>VEGF anti-sense</td>
<td>5'-ACT CAA CTA GGT AGT CAG TCA GG-3'</td>
<td></td>
</tr>
<tr>
<td>(\beta)-actin sense</td>
<td>5'-TCA TGA GGT AGT CAG TCA GG-3'</td>
<td></td>
</tr>
<tr>
<td>(\beta)-actin anti-sense</td>
<td>5'-TCA TGA GGT AGT CAG TCA GG-3'</td>
<td></td>
</tr>
</tbody>
</table>
It was necessary to show the differential effect of hypoxia on MMP production in RA FLSs.

Differential effect of hypoxia on MMP production depended on HIF-1α expression in IL-1β-stimulated RA FLSs. HIF-1α expression in rheumatoid synovial fibroblasts is known to be increased by both pro-inflammatory cytokines and hypoxia [19]. We tested whether the expression of HIF-1α would be increased in RA FLSs by either IL-1β or hypoxia stimulation in this system. We found that the expression of HIF-1α in RA FLS was greatly increased by IL-1β or hypoxia (Fig. 3A). To determine whether the differential effect of hypoxia on the production of MMPs was dependent on HIF-1α expression, we knocked out the expression of HIF-1α in synovial cells by transfection with HIF-1α siRNA. The cells transfected with siRNA against HIF-1α or control siRNA were stimulated with IL-1β (10 ng/ml) under hypoxia or normoxia. Supernatants were collected for ELISA and the cells were used for RNA extraction and expression analysis. Three independent experiments were performed in quadruplicate with FLSs from each patient. Similar results were obtained for all three, and the data shown are representative. Values are expressed as mean (S.E.M.). **P < 0.01 and ***P < 0.001 vs the normoxic group.
IL-1β-stimulated RA FLSs was entirely dependent on HIF-1α expression. Also, it provided indirect evidence that HIF-1α expression was not significantly involved in the IL-1β-stimulated increase in MMP expression in RA FLS under normoxic conditions.

**Discussion**

In this study, we showed that hypoxia differentially regulated the effects of IL-1β on MMP-1 and MMP-13 expression. Under hypoxic conditions, the increased expression of MMP-1 by IL-1β was further increased, but the increased expression of MMP-13 by IL-1β was reduced. When HIF-1α was knocked out with siRNA, the differential effect of hypoxia on IL-1β-mediated MMP expression was significantly attenuated. This suggested that HIF-1α was a key player in the regulation of this differential effect on MMP expression.

Regulation of MMP gene expression can be achieved at multiple levels [20]. MMP gene expression is, to a large extent, regulated at the transcriptional level, but it is also controlled at the post-transcriptional level. In addition, it is controlled by promoter polymorphisms and epigenetic regulation. In particular, some MMPs appear to be distinctly expressed in specific cell types or tissues due to the restricted expression of transcription factors in that particular cell type. For example, MMP-28 is exclusively expressed in developing germ cells by the induction of SOX-5 (SRY-related HMG-box) [21]. MMP-13 is almost exclusively expressed in developing cartilage and bone by the induction of Runx domain factor-2 (Runx-2) [12]. Among MMPs, a Runx-2 binding site is unique to the MMP-13 promoter, and it cooperates with an activator protein 1 (AP-1) domain to mediate MMP-13 transcription. However, MMP-13 expression also requires nuclear factor-kappa B (NF-κB) activation in combination with inflammatory stimuli, such as IL-1β and TNF-α [22, 23]. This was demonstrated by the fact that inhibiting NF-κB activation caused more effective inhibition of MMP-13 than MMP-1 expression with IL-1β stimulation in FLSs or chondrocytes [18, 24]. That suggested that NF-κB activation was more important than Runx-2 activation for the expression of MMP-13 in IL-1β-stimulated RA FLS. In addition, an AG-rich element (AGRE) regulatory site was recently found in the proximal promoter of the human MMP-13 gene [25]. The complicated transcriptional regulation of MMP-1 and MMP-13 genes has been reviewed previously [12, 26, 27].

Hypoxia is known to up-regulate angiogenesis and synovial cell migration in RA by inducing MMPs and VEGF [2]. In particular, hypoxia-induced MMP-13 expression may enhance the permeability of endothelial cells in RA [28]. HIF-1α has been shown to play key roles in increasing the expression of various genes that contribute to angiogenesis, oxygen transport, glucose metabolism, vascular tone,
and cell proliferation and survival [29, 30]. Ahn et al. [31] showed that hypoxia-induced MMP-3 expression was significantly attenuated by knock-down of HIF-1α. Thus, HIF-1α is a promising therapeutic target in RA. Silencing HIF-1α also inhibited cell migration and invasion under a hypoxic environment in malignant gliomas [32]. However, hypoxia accelerated cancerous invasion of hepatoma cells by up-regulating MMP expression in a HIF-1α-independent manner [33].

IL-1β also stimulates HIF-1α expression through extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3 kinase (PI3K) pathways [19]. Therefore, hypoxia-regulated gene expression is further increased by IL-1β-induced HIF-1α expression. This was supported by our finding that the differential effect was not apparent at low concentrations of IL-1β (0.1 ng/ml). This suggested that a minimal level of HIF-1α was required for a further increase in MMP-1 expression. However, the increase in MMP-13 expression induced by IL-1β stimulation was decreased by hypoxia; this suggested indirectly that the regulation of MMP-13 gene expression may be much more complicated than that of MMP-1.

The exact mechanism by which hypoxia affects IL-1β stimulation of MMP expression is unclear. A recent report showed that, among 100 transcription factors, both HIF-2α and HIF-1α were the strongest activators of the Runx2-P1 promoter activity, and the Runx2 binding site is unique to the promoter for MMP-13 [34]. When siRNA was used to knock-down HIF-2α, they found that HIF-2α was an important regulator of Runx2 gene expression. However, in our system, the knock-down of HIF-1α did not significantly decrease the level of Runx2 gene expression.

**Fig. 3** Effect of HIF-1α knock-down on the differential expressions of MMP-1 and MMP-13 stimulated by IL-1β under hypoxia. (A) Increased HIF-1α expression under hypoxia or IL-1β stimulation, detected by western blot. (B) Cells were transfected with siRNA against HIF-1α or control siRNA. The levels of HIF-1α expression after siRNA transfection were determined by western blot. (C) The levels of VEGF, MMP-1 and MMP-13 in culture supematants of the transfected cells were determined by ELISA. Three independent experiments were performed in quadruplicate. Each experiment was performed using synovial cells from different patients. The data shown are representative of three independent experiments, and similar results were obtained from all three. Values are expressed as mean (±S.E.M.). *P < 0.05 vs the normoxic group.
expression (data not shown); this suggested that transcription factors other than Runx2 might be involved in the hypoxia-induced reduction of inflammatory factor-stimulated MMP-13 expression.

In conclusion, hypoxia in arthritic joints may induce the differential expression of MMP-1 and MMP-13 in a HIF-1α-dependent manner in rheumatoid synovial fibroblasts during joint inflammation. In particular, hypoxia increased MMP-1 and reduced MMP-13 stimulation by the inflammatory cytokine, IL-1β. Therefore, this differential effect mediated by hypoxia should be taken into consideration when testing the efficiency of therapies that target HIF-1α to control genes important in bone degradation in RA.

**Rheumatology key messages**

- IL-1β stimulation under hypoxia induced an MMP-1 increase and an MMP-13 decrease in FLSs.
- HIF-1α knock-down may increase the expression of other genes in therapies using siRNA.

**Acknowledgements**

_Funding:_ This work was supported by a grant from the Kyung Hee University in 2010 (KHU-20100691).

_Disclosure statement:_ The authors have declared no conflicts of interest.

**References**

25. Fan Z, Tardif G, Boileau C et al. Identification in human osteoarthritic chondrocytes of proteins binding to the novel regulatory site agr-e in the human matrix


450