Influence of hypoxia on the expression of matrix metalloproteinase-1, -3 and tissue inhibitor of metalloproteinase-1 in rheumatoid synovial fibroblasts

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Abstract

Objective

The rheumatoid synovium is a hypoxic environment, and hypoxia has been implicated as a factor in the pathogenesis of rheumatoid arthritis (RA). The purpose of this study was to investigate the effect of hypoxia on the expression of matrix metalloproteinase (MMP)-1, -3 and tissue inhibitor of metalloproteinase (TIMP)-1 in rheumatoid synovial fibroblasts.

Methods

Synovial fibroblasts obtained from RA patients were cultured for 48 h under normoxic or hypoxic conditions. Assays included western blot analysis and enzyme-linked immunosorbent assay (ELISA) for MMP-1, -3 and TIMP-1, and northern blot analysis to measure TIMP-1 mRNA levels.

Results

Compared with normoxic culture, hypoxia increased MMP-1 and MMP-3 expression in rheumatoid synovial fibroblasts. Hypoxia decreased TIMP-1 expression in rheumatoid synovial fibroblasts, as measured by both protein and mRNA levels.

Conclusion

These results suggest that microenvironmental conditions, such as hypoxia, may directly contribute to joint destruction in RA by increasing the ratio of MMP-1, -3 to TIMP-1 production in synovial fibroblasts.

Key words

Hypoxia, rheumatoid arthritis, matrix metalloproteinase, tissue inhibitor of metalloproteinase.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder of unknown etiology. The rheumatoid joint is characterized by the tumor-like growth of synovial fibroblasts, called pannus, and invasion of the pannus into adjacent cartilage and bone. The rheumatoid synovium is a hypoxic environment due to increased oxygen consumption by metabolically active tissue (1), and because, during movement, intra-articular pressure in the chronically inflamed joint exceeds capillary perfusion pressure, resulting in impaired oxygen supply to the synovium (2). This hypoxic microenvironment of the joint has been implicated in the pathogenesis of RA and osteoarthritis. It is thought that hypoxia is one of the major contributors to angiogenesis in rheumatoid synovium by inducing vascular endothelial growth factor (VEGF) secretion in synovial fibroblasts (3). New blood vessel formation is important to maintaining the pannus mass and to recruiting inflammatory cells to the diseased synovium (4). Recently, hypoxia has been shown to modulate expression of various genes and production of proinflammatory mediators in articular chondrocytes, suggesting that a hypoxic environment contributes to the pathogenesis of osteoarthritis (5).

Matrix metalloproteinases (MMPs) are responsible for the destruction of cartilage and bone in the inflamed joint, and MMPs are counter-regulated by tissue inhibitor of metalloproteinases (TIMPs). In particular, collagenase 1 (MMP-1) and stromelysin 1 (MMP-3) are important proteases in tissue degradation in RA; these enzymes are inhibited by TIMP-1 (6). The hypoxic environment might affect matrix degradation by controlling expression of these MMPs or TIMP-1. Using *in vitro* cell culture studies, we investigated the effect of hypoxia on expression of MMP-1, -3 and TIMP-1 in rheumatoid synovial fibroblasts.

Materials and methods

Primary synovial fibroblast culture

The experimental protocol was approved by the local ethics committee and a signed consent form was obtained from each patient. Patients with RA were diagnosed according to the standards of the American College of Rheumatology (7). Synovial tissue, obtained from RA patients undergoing total knee joint replacement surgery, was minced, digested overnight with 5 mg/ml type IV collagenase (Sigma, Poole, UK) and 150 µg/ml type I DNase (Sigma), and separated from undigested tissue by unit gravity sedimentation. After collecting the suspended cells into fresh tubes, the cells were harvested by centrifugation at 500 µg for 10 min. The pellet was washed twice with Dulbecco's Modified Eagle's Media (DMEM; Life Technologies, NY, USA) containing 10% fetal calf serum (FCS). Resuspended cells were plated at a concentration of 2 x 10^6/ml in a total volume of 1 ml/200 mm^2 into T-25 culture flasks. After overnight incubation, non-adherent cells were removed by replacing fresh culture medium, and attached cells were cultured in DMEM with 10% FCS and 50 units/ml penicillin, 50 mg/ml streptomycin, and 0.025 mg/ml amphotericin B until 90% confluent growth. Primary cultured cells were passaged three to four times over several weeks for subsequent experiments.

Synovial fibroblasts cultured under hypoxic conditions

For each experiment, cells were incubated under hypoxic conditions using a BBL® Gaspak pouch™ anaerobic system containing anaerobic indicator (Becton-Dickinson, Lincoln Park, NJ, USA) as previously described (8). The medium was changed to serum-free medium just before culturing synovial fibroblasts in the hypoxic condition. Each Gaspak pouch system produced an anaerobic condition, with an oxygen concentration of less than 2% and a carbon dioxide concentration of greater than 4% achieved within 1 h of incubation at 37ºC. In each experiment, parallel cultures were performed in serum-free medium placed in a normoxic condition in a 5% CO_2 incubator (SANYO Co., Japan). After culturing synovial

fibroblasts under these conditions for 48 h, cells were harvested for experiments. Cell viability was determined by trypan blue exclusion.

Enzyme-linked immunosorbent assay (ELISA)

The expression levels of MMP-1, MMP-3, and TIMP-1 were measured in primary cultured rheumatoid synovial fibroblasts growing under hypoxic conditions. The culture medium was replaced with serum-free DMEM medium supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.025 μg/ml amphotericin B, and cells were incubated at 37ºC, in a humid atmosphere of 5% CO_2/95% air for 48 h. After incubation, the supernatants were collected, and then centrifuged to remove cell debris. Expression of MMP-1, MMP-3, and TIMP-1 in supernatants was quantified using commercially available ELISA kits according to the manufacturer's instructions (Oncogene™ research products, USA).

Western blot analysis

Whole cell lysates (WCL) were prepared using RIPA buffer containing 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 BAC protein assay kit with bovine serum albumin as the standard. Fifteen microliters (50 μg/ml) of WCL were separated by electrophoresis on a 10% acrylamide gel with a constant of 50 V for 3 h. The separated proteins were transferred electrophoretically onto nitrocellulose membranes. Membranes were preblocked 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 5% skim milk powder. Membranes were incubated overnight at 4ºC with 1:500-diluted monoclonal antibodies to MMP-1, MMP-3, or TIMP-1 (Santacruz Co., 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 or anti-rabbit IgG antibody (Amersham, USA). Proteins were detected using enhanced chemiluminescence (Western blot analysis system; Amersham).

Cloning DNA and Northern blot analysis

Total RNAs were isolated from primary cultured synovial fibroblasts using TRIzol containing phenol and guanidine isothionate in a monophase solution, as described in the manufacturer's protocol (Gibco BRL, USA). Total RNA (20 μg per lane) was denatured in 50% formamide containing 7.4% formaldehyde, and separated by electrophoresis on an agarose gel containing 1% agarose/2.2 M formaldehyde. To confirm RNA integrity and equal quantities, ethidium bromide staining of 18S and 28S ribosomal RNA bands was performed. The RNA was blotted by capillary action onto Hybond membrane (Amersham) in 10 x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). For preparing probes for TIMP-1 and GAPDH, RT-PCR was performed using Superscripts II (Gibco BRL) according to the manufacturer's instructions. Two oligo primers were synthesized based on the reported sequences of TIMP-1 (Access# AF366397) (5'-ATGGCCCCCTTT-GAGCCCCTG-3' and 5'-TCAGGC-TATCTGGGACCGCAG-3') and GAPDH (Access# M33197) (5'-TGATTCCACCCATGGCAAATT-3' and 5'-CAGTCTTCTGGGTGGCAG-TG-3'). For both TIMP-1 and GAPDH, cDNAs were amplified by 30 cycles and the thermal cycle profile was as follows: an initial denaturation at 95°C for 5 min, a denaturation step at 95°C for 30 s, an annealing step at 56°C for 1 min, and an extension step at 72°C for 3 min., a final extension at 72°C for 10 min. After the reaction, the amplified DNAs were separated onto 1% agarose gel and the DNA fragments isolated using the Gel Extraction Kit (GENOMED Co. USA). Amplified DNA was subcloned into a Topo cloning vector (Invitrogen. Co. USA), and cloned DNA was confirmed by DNA sequencing. Probes were prepared by restriction digestion of the Topo cloning vector (Invitrogen Co. USA) with *Eco*RI

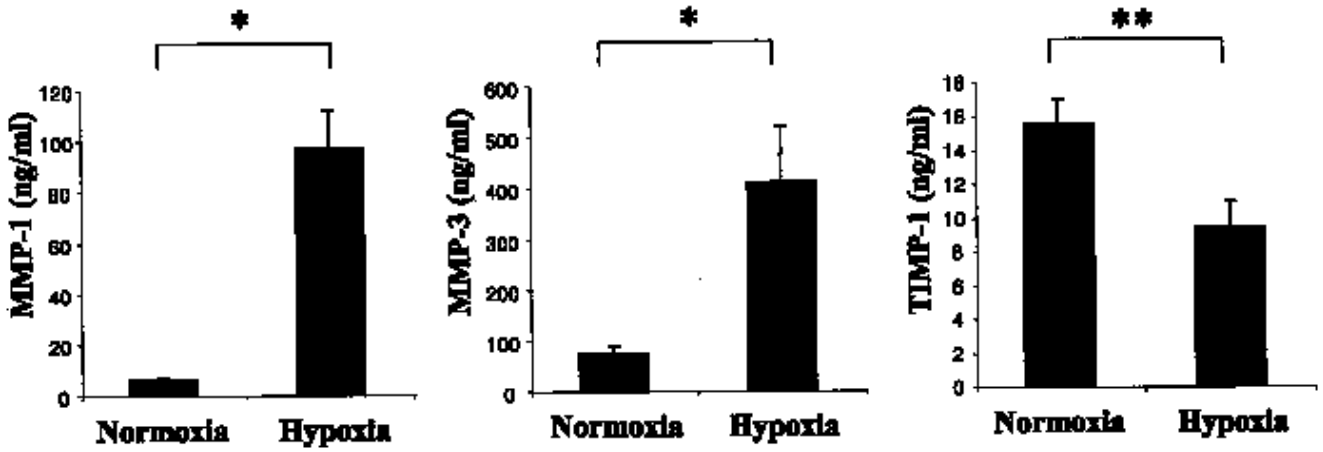

Fig. 1. MMP-1, -3,and TIMP-1 production by rheumatoid synovial fibroblasts in the hypoxic condition. Synovial fibroblasts obtained from 5 RA patients were incubated under normoxic or hypoxic conditions for 48 h. Concentrations of MMP-1,-3,and TIMP-1 in the supernatants were determined using ELISA. Data are presented as mean ± SEM. $^{*}P < 0.01$, $^{**}P < 0.05$.

followed by isolation of the insert band on 1% agarose, and probes for TIMP-1 gene (623 bp) and GAPDH (415 bp) were then labeled with α-^{32}P-dCTP using the random hexamer-primed method (Roche, Germany). Following hybridization to the probe at 42°C, the blots were washed twice in 2 x SSC containing 0.5% SDS at room temperature, and then exposed on x-ray film. The expression level of the TIMP-1 gene and GAPDH was measured using densitometry (GS-800 calibrated densitometer; BioRad, USA).

Statistical analysis

Statistical analysis was performed using the SPSS statistical package (version 10.0 for Windows; SPSS, Chicago, IL). Data are expressed as the mean ± SEM. Comparisons among groups were performed by a Mann-Whitney test. A 5% significance level was accepted as showing statistical significance.

Results

In the first experiment, synovial fibroblasts from 5 RA patients were cultured under normoxic or hypoxic conditions for 48 h, and then ELISA for MMP-1, MMP-3, and TIMP-1 was performed on supernatants from these cells. Levels of MMP-1 and MMP-3 were significantly higher in the hypoxic than the normoxic condition ($P < 0.01$). In contrast, the TIMP-1 level was significantly lower in the hypoxic than in the normoxic condition ($P < 0.05$) (Fig. 1).

Western blot analysis showed that hypoxic culture resulted in a significant increase in MMP-1 protein expression in the WCL of rheumatoid synovial fibroblasts ($P < 0.05$). There was a non-significant trend for increased MMP-3 protein expression under hypoxia. In contrast, TIMP-1 protein expression significantly decreased under the hypoxic compared with the normoxic condition ($P < 0.01$) (Fig. 2).

To confirm the hypoxia-induced decrease of TIMP-1 at the transcriptional level, Northern blot analysis was performed in rheumatoid synovial fibroblasts from six RA patients. As shown in Figure 3, there were marked decreases of TIMP-1 mRNA levels in rheumatoid synovial fibroblasts cultured under the hypoxic compared with the normoxic condition ($P < 0.01$). A known constant that is independent of O_2 tension, 28S rRNA, was used in all cultures (9). In addition, the expression of GAPDH was examined as a hypoxic indicator, since the GAPDH gene is known to be up-regulated under hypoxic conditions by inhibiting the TCA cycle (9). In our experiment, as expected, hypoxia increased the expression of GAPDH in synovial fibroblasts.

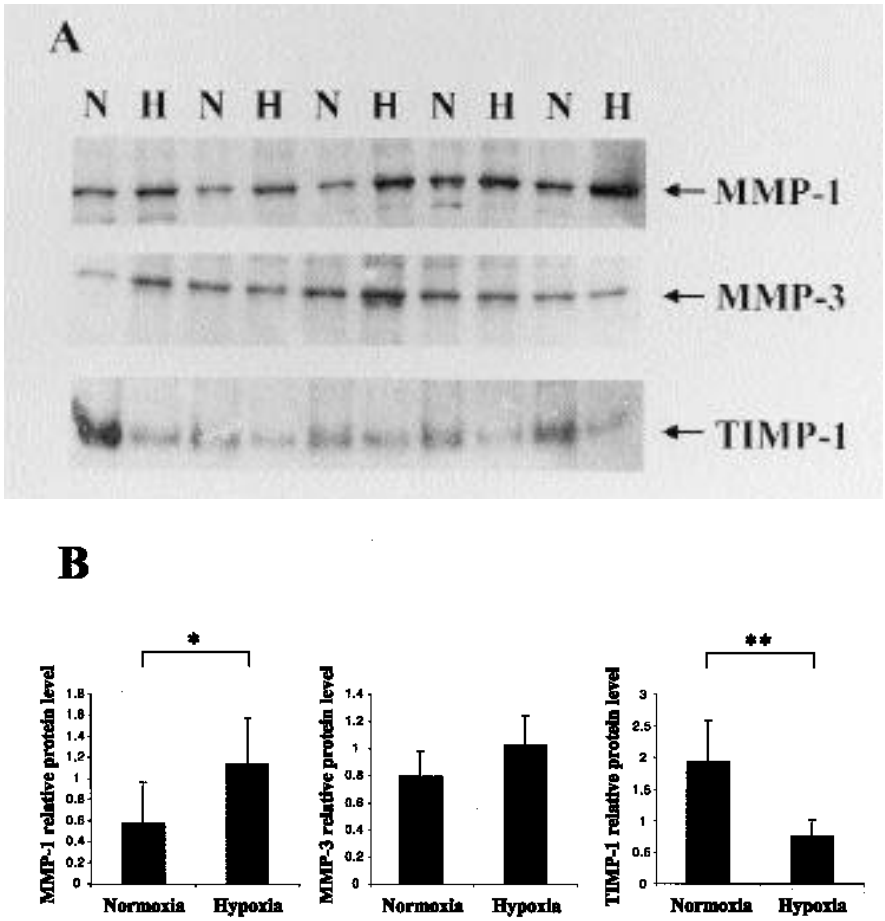

Fig. 2. MMP-1,-3, and TIMP-1 protein expression in rheumatoid synovial fibroblasts in hypoxic culture. (**A**) After synovial fibroblasts from five RA patients were incubated under normoxic (**N**) or hypoxic (**H**) conditions for 48 h, whole cell lysates were subjected to Western blots of MMP-1,-3,and TIMP-1. (**B**) Relative protein levels were measured by densitometry. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

Discussion

Our findings indicate that, in synovial fibroblasts, hypoxia can directly affect joint destruction in inflammatory arthritis by regulating the expression of MMPs and TIMP-1. Western blot analysis of MMP-3 conducted on cell lysates did not show a clear increase under hypoxia but the levels of both MMP-1 and MMP-3 were significantly increased in the culture medium under hypoxia. This result implicates that hypoxic stress may at least stimulate MMPs secretion from rheumatoid synovial fbroblasts even though transcriptional activations of MMPs are not apparently affected. Furthermore, what we think is more important in this study is the consistent down-regulation of TIMP-1 by hypoxia. The marked decrease of TIMP-1 expression without accompanying decrease of MMPs expression by hypoxia might facilitate matrix degradation in the rheumatoid joint.

In the rheumatoid synovium, fibroblast-like synoviocytes play an essential role in the destruction of cartilage and bone, by producing most of the degradative enzymes such as collage-

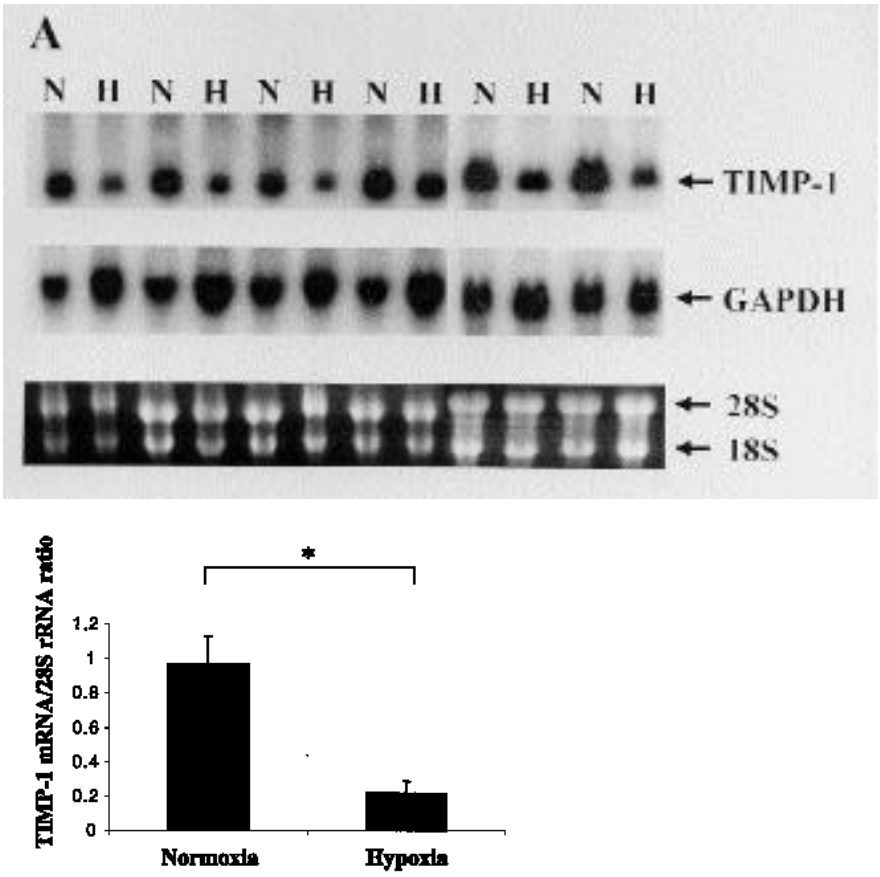

Fig. 3. Effect of hypoxia on TIMP-1 mRNA expression in rheumatoid synovial fibroblasts. (**A**) Synovial fibroblasts obtained from six RA patients were cultured under normoxic (**N**) or hypoxic (**H**) conditions for 48 h. Twenty micrograms of total RNA isolated from the cell cultures were loaded per lane and analyzed by Northern blotting. To confirm the hypoxic condition, GAPDH expression was examined under the same condition. 18S and 28S rRNA were used to normalize the equal loading amounts. (**B**) Quantitative analysis of TIMP-1 mRNA and 28S rRNA was performed by densitometry. The results are expressed as the ratio of TIMP-1 mRNA to 28S rRNA. Data are presented as mean ± SEM. $^{*}P < 0.01$.

nase, stromelysin, and the cathepsins (10). These degradative proteases are constitutively expressed in resting fibroblasts; on exposure to cytokines such as interleukin-1 or tumor necrosis factor-α, however, their production increases (11). In this study, to exclude the possible influence of serum factors such as cytokines on expression of MMPs or TIMP-1, we used serum-free media for all experiments. Both proinflammatory cytokines and a hypoxic microenvironment may be important to the role of synovial fibroblasts in the pathophysiology of RA. There is evidence of synovial hypoxia in RA. First, anaerobic metabolism via the glycolytic pathway increases in rheumatoid synovial tissue (1, 12, 13). Second, it has been reported that oxygen tension measured directly in synovial fluid or tissue was lower in RA patients than in a variety of other conditions (14, 15). Third, recent data indicate that hypoxia-inducible factor 1α (HIF-1α) expression is specifically up-regulated in RA synovia compared with osteoarthritis or healthy synovia (16).

Previously reported effects of hypoxia on the production of MMPs or TIMPs are variable, depending on cell types and experimental conditions. For example, low oxygen tension increased gelatinase activity in human trophoblasts (17), and hypoxia induced enhanced secretion of the 92-kDa type IV collagenase in human keratinocytes (18). Increased exposure to hypoxic conditions also increased the 92-kDa collagenase in cardiomyocytes (19). In contrast, hypoxia did not up-regulate MMP-9 in various tumor cell lines except the alveolar rhabdomyosarcoma cell line (20); in normal human dermal fibroblasts, MMP-3 was down-regulated under hypoxic conditions (21). Hypoxia induced a time-dependent increase in TIMP-1 mRNA levels in kidney fibroblasts (22). Rheumatoid synovial fibroblasts are known to have partially transformed characteristics that distinguish rheumatoid synovial fibroblasts from other cells (11). Reactive oxygen species and reactive nitrogen species are thought to be the possible cause of somatic mutations in RA; in inflamed synovium, reoxygenation after hypoxia might account for these oxidative stresses (11). Hence, it is possible that rheumatoid synovial fibroblasts require hypoxic stimuli to exert aggressive features (23).

The mechanism by which hypoxia affects the expression of MMPs or TIMP-1 is unclear. HIF-1α, a common transcription factor induced by hypoxia,might mediate the gene transcription of MMPs or TIMP-1. On the other hand, hypoxia would stimulate cytokine production in rheumatoid synovial fibroblasts, which in turn might regulate the production of MMPs and TIMP-1 in synovial fibroblasts in an autocrine or paracrine fashion. Indeed, hypoxia has been shown to induce transcriptional activation of genes encoding growth factors and cytokines such as endothelin-1 (24), platelet-derived growth factor (25), interleukin-1 (26), and tumor necrosis factor-α (27, 28) in endothelial cells or mononuclear cells. Although the influence of hypoxia on synovial fibroblast cytokine production is unknown at present, it is possible that cytokines participate in this mechanism.

In conclusion, our results show that hypoxia, which occurs in the rheumatoid synovium, directly increases synovial fibroblast production of MMP-1 and MMP-3, and decreases production of TIMP-1. Considering that the balance between MMPs and TIMPs determines tissue degradation, such up-regulation of MMPs and down-regulation of TIMP-1 in synovial fibroblasts by hypoxia may facilitate destruction of cartilage and bone.

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