Hypoxia inducible factor-1 alpha expression in human normal and osteoarthritic chondrocytes

Ibsen B. Coimbra M.D., Ph.D., Sergio A. Jimenez M.D., David F. Hawkins, Sonsoles Piera-Velazquez Ph.D. and David G. Stokes Ph.D.*
Department of Medicine, Division of Rheumatology, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA 19107, USA

Summary

Background: Articular cartilage is a unique tissue in that it is avascular with its nutrition and oxygen supply being dependent on the diffusion of solutes through the synovial fluid and to and from the subchondral bone. The oxygen levels in articular cartilage, therefore, are assumed to be low. Oxygen is an important modulator of gene expression and this regulation occurs largely through the activation of the transcriptional complex hypoxia-inducible factor-1 (HIF-1). However, little is known about how articular cartilage regulates genes in response to O2 tension and whether this regulation occurs through HIF-1.

Aim: The aim of this study was to investigate the expression profile of HIF-1α in normal and osteoarthritic (OA) chondrocytes under normoxic and hypoxic conditions, and in response to treatment with tumor necrosis factor alpha (TNFα).

Methods: Articular chondrocytes from human normal and OA knee cartilage were isolated and cultured in suspension under normoxic (21% O2) or hypoxic conditions (1% O2). Chondrocytes were also treated with TNF-α under normoxic conditions. Nuclear extracts and total RNA were prepared and HIF-1α protein and mRNA levels were assayed by immunoblotting and Northern hybridization. Localization of HIF-1α by immunofluorescence was performed on frozen sections of cartilage tissue by confocal microscopy.

Results: HIF-1α expression was detectable in human normal and OA chondrocytes and cartilage by Northern analysis, immunoblotting and immunofluorescence under normoxic conditions. Culture of OA or normal chondrocytes under hypoxic conditions for up to 16 h resulted in a modest stabilization and/or increase of HIF-1α expression. Treatment of articular chondrocytes with TNFα resulted in an increase in HIF-1α protein steady state levels under normoxic conditions. The increase in HIF-1α expression induced by TNFα was partially blocked by pretreatment of the chondrocytes with inhibitors of NFκB or p38 MAP kinase. We also observed the expression of HIF-2α mRNA in human chondrocytes.

Conclusion: HIF-1α is expressed in human normal and OA articular chondrocytes cultured under normoxic conditions. HIF-1α can be further induced or stabilized in articular chondrocytes by hypoxia or by treatment with TNFα. The relatively high constitutive expression of HIF-1α by chondrocytes may be an important adaptation to survival in the avascular-hypoxic environment of cartilage. Modulation of HIF-1α levels by TNF-α may have important implications for chondrocyte metabolism during degenerative joint disease. In addition, we detected for the first time the expression of HIF-2α mRNA in chondrocytes.

Key words: Gene expression, Human chondrocytes, Hypoxia, Osteoarthritis, Articular cartilage.

Introduction

It is well known that articular cartilage is an avascular tissue that derives its nutrition and oxygen supply by diffusion from the synovial fluid and the subchondral bone. It has been estimated that the chondrocytes at the articular surface are exposed to approximately 6 to 10% O2 whereas the chondrocytes in the deepest layers of the articular cartilage may have access to only 1 to 6% O2 or less1–7.

Other studies have shown that articular chondrocytes derive up to 75% of their ATP requirement from anaerobic glycolysis, underlining their adaptation to a low O2 environment8,9. However, the mechanisms by which articular chondrocytes regulate genes that are sensitive to O2 levels under normal and pathological conditions remain poorly understood.

The role of oxygen as an important modulator of gene expression is well recognized and extensive evidence implicates hypoxia-inducible factor (HIF-1) as the primary gene regulatory factor that responds to variations in O2 levels10. HIF-1 is a heterodimer consisting of two different subunits, HIF-1α and HIF-1β (also known as ARNT-aryl-hydrocarbon receptor nuclear translocator) that are members of the bHLH-PAS (basic helix-loop-helix-PER-ARNT-SIM) family of transcription factors11,12. HIF-1β is constitutively expressed, whereas exposure to hypoxia inhibits the rapid proteosome-mediated degradation of HIF-1α, thereby increasing the steady-state levels of HIF-1α protein.
in the cell. Once HIF-1α protein levels are stabilized, it can translocate to the nucleus, dimerize with HIF-1β and bind to regulatory regions of hypoxia-responsive genes through the hypoxia response element (HRE) leading to their transcriptional activation. Genes that are regulated by HIF-1 in response to O2 tension include those encoding growth and angiogenic factors, glycolytic enzymes and glucose transporters. Indeed, it has been demonstrated that mammalian cells lacking HIF-1α show decreased growth rates during both hypoxia and normoxia, as well as decreased levels of mRNA for multiple glycolytic enzymes and glucose transporters. The diminishing glycolytic capacity results in lowered free ATP levels in hypoxic cells and, therefore, HIF-1 activation is an important control mechanism of the metabolic state during hypoxia. It has become increasingly clear, however, that HIF-1α levels are regulated by factors other than O2 levels. Recent work has shown that growth factors, such as IGF-1 and 2, and cytokines, such as IL-1 and TNFα, can influence the levels of HIF-1α protein and HIF-1 DNA-binding activity within the cell. Interestingly, both cytokines and growth factors generally cause an induction of HIF-1α levels or HIF-1 DNA-binding activity, albeit most likely through different mechanisms. Indeed, recent evidence indicates that IGF-1 acts to increase the levels of HIF-1α by influencing the translation of HIF-1α mRNA whereas TNFα may regulate HIF-1α by increasing its stability and nuclear translocation in a NF-κB dependent manner.

Since it is assumed that articular cartilage is normally hypoxic as compared to vascularized tissues, there has been investigation into the effect of low O2 tension on the growth and phenotype of articular cartilage in vitro. These studies have substantiated the notion that chondrocytes are well adapted to an hypoxic environment and that culture in a low O2 tension environment may be beneficial for maintenance of the chondrocyte-specific phenotype in vitro. Indeed, culture of articular chondrocytes in a low O2 environment has been shown to augment chondrocyte-specific gene expression and speed recovery of the differentiated phenotype following loss of their chondrocyte-specific phenotype during monolayer culture. It has been postulated that during degenerative joint diseases such as OA, the O2 gradient across articular cartilage may be altered due to cartilage thinning and erosion, changes in extracellular matrix composition, and the development of cartilage fissures. However, very little is known about how articular chondrocytes regulate gene expression in response to varying O2 concentrations under normal and pathological situations, and to what extent HIF-1 is involved in the response. We recently reported that HIF-1α mRNA levels are down-regulated in de-differentiated chondrocytes as compared to differentiated chondrocytes and we have also detected HIF-1α transcripts in both human adult normal and OA chondrocytes. The aim of the present study was to investigate the expression profile of HIF-1α in normal and OA human chondrocytes under normoxic and hypoxic conditions and in response to treatment with TNFα and to investigate the localization of HIF-1α in human articular cartilage.

Materials and methods

ISOLATION AND CULTURE OF HUMAN NORMAL AND OA CHONDROCYTES

Human chondrocytes were obtained from the femoral condyles and tibial plateaus of patients with OA that underwent knee-joint replacement surgery at Thomas Jefferson University Hospital and from normal donors at autopsy through the National Disease Research Interchange or the Cooperative Human Tissue Network following procedures approved by the Institutional Review Board. The chondrocytes were isolated from all remaining cartilage tissue as previously described. Briefly, to remove adherent fibrous tissues, the cartilage was incubated in Hanks’ medium containing trypsin and bacterial collagenase (2 mg/ml each) for 1 h at 37°C. The medium was discarded and the tissue fragments minced and digested overnight at 37°C in Dulbecco’s minimum essential medium (DMEM) with 4.5 g/l glucose containing 10% fetal bovine serum and 0.5 mg/ml bacterial collagenase. The released cells were filtered through a 70 µm nylon cell strainer and were collected by centrifugation at 250×g for 5 min and washed four times with collagenase-free medium. Isolated chondrocytes were immediately frozen in freezing media (90% FBS, 10% DMSO) and stored for future experiments. For experiments involving suspension cultured chondrocytes, which preserves the differentiated phenotype, cells were thawed and plated into suspension in 6-well ultralow attachment plates (Corning, Acton, MA) at a density of 1×10⁶/ml. The cells were allowed to recover for 48 h at which time they were utilized for experiments. Chondrocytes utilized for experiments in suspension culture were not cultured in monolayer before use. For experiments involving monolayer cultures of chondrocytes, cells were thawed and plated into 162 cm² plastic tissue culture flasks at a density of 30 000/cm² and cultured for 30 days through three passages. All cultures were grown in DMEM containing 10% FBS, 2 mM glutamine, 1% vitamin supplements, 100 units/ml penicillin and 100 µg/ml streptomycin. Amphotericin B and ascorbic acid were avoided because they can interfere with HIF-1α expression. For normoxic conditions, cells were maintained at 37°C in 5% CO2 and 95% air (21% O2). To subject the cells to hypoxia, cultures were placed in a modular incubator chamber (Billups-Rothenburg, Del Mar, CA) that had been flushed with a mixture of 1% O2, 5% CO2 and 94% N2. Hypoxic conditions within the chamber were verified with a YSI model 55 dissolved oxygen meter (YSI Inc., Yellow Springs, OH). TNFα was purchased from Pierce Endogen (Rockford, IL). For TNFα treatment, chondrocytes were cultured for 18 h in media containing 0.5% serum followed by a 4 h treatment with 10ng/ml TNFα in media containing 0.5% serum.

RNA ISOLATION AND NORTHERN HYBRIDIZATION ANALYSIS

Total cellular RNA was isolated from human HTB chondrosarcoma cells and human normal and OA chondrocytes utilizing the Trizol reagent (Life Technologies, Rockville, MD) according to the manufacturer’s specifications. HTB chondrosarcoma poly-A+ RNA was obtained using the Oligotex mRNA kit from Qiagen (Valencia, CA). cDNA for human HIF-1α, -2α and -1β was generated using Superscript II reverse transcriptase (Life Technologies) and oligo-dT as a primer. PCR was performed with the Expand High Fidelity Polymerase System (Roche Diagnostics, Indianapolis, IN) and PCR products were cloned into the pCR-Blunt vector (Invitrogen, Carlsbad, CA). The following primer pairs were used: HIF-1α, ACCATGCCCCAGATTCCGGA (forward) and ATCAGTGGTGCGACTGATGTT (reverse);
DIFFERENTIATED CHONDROCYTES EXPRESS HIF-1α PROTEIN UNDER NORMOXIC CONDITIONS

Normally, HIF-1α protein levels are undetectable in cells under normoxic conditions, however, the expression profile of HIF-1α in articular cartilage, an avascular-hypoxic tissue, is not known. In order to determine whether articular chondrocytes express HIF-1α protein under normoxic conditions, nuclear extracts were prepared from human OA chondrocytes. I. B. Coimbra et al. HIF-1α expression in human articular chondrocytes.
chondrocytes cultured either in suspension (differentiated) or as monolayers in plastic tissue culture flasks (de-differentiated) and then Western blot analysis was performed with anti-HIF-1α antibodies. Surprisingly, we detected a strong HIF-1α signal in differentiated articular chondrocytes cultured in suspension under normoxic conditions (Fig. 2A). In agreement with our earlier studies in which we observed a down-regulation of HIF-1α mRNA in de-differentiated chondrocytes, we could not detect HIF-1α protein by Western analysis in de-differentiated human OA chondrocytes (Fig. 2A). In order to verify our observation of HIF-1α expression in chondrocytes under normoxic conditions, we performed Western analysis on nuclear extracts from chondrocytes isolated from six other donors; four from patients with OA and two normal donor samples. We detected HIF-1α expression in all six samples as can be seen in the Western analyses shown in Fig. 2B.

INDUCTION OF HIF-1α IN ARTICULAR CHONDROCYTES BY HYPOXIA

We next determined whether hypoxia would induce higher levels of HIF-1α in OA or normal articular chondrocytes in culture. Utilizing a hypoxic incubation chamber we first determined the dissolved O2 levels in cell culture media after purging the chamber with a mixture of 94% N2, 5% CO2 and 1% O2. As can be seen in Fig. 3A, hypoxic conditions in the media were achieved within 1 h of purging and remained stable for up to 16 h. This was further verified by subjecting HEP-3B cells to a 4 h hypoxic treatment in the chamber followed by immunofluorescence to detect HIF-1α expression (Fig. 3B). OA or normal articular chondrocytes that were cultured in suspension were subjected to a hypoxic treatment of 1% O2 for 0, 4, 8 or 16 h. Nuclear extracts were then prepared and HIF-1α protein levels were determined by Western analysis. As can be seen in Fig. 3C-E, exposure of either normal or OA articular chondrocytes to hypoxic conditions for increasing lengths of time resulted in a modest increase in the HIF-1α signal. We also observed that the HIF-1α bands became consolidated into a single higher molecular weight band during the hypoxic treatment. Whether this is due to a decrease in degradation, an increase in phosphorylation, or both, remains to be determined. Finally, as a control for overall HIF-1α induction, we performed an induction experiment with the hypoxia mimic CoCl2. As can be seen in Fig. 3F, HIF-1α is readily inducible in normal human articular chondrocytes upon a 4 h treatment with 100 µM CoCl2.

LOCALIZATION OF HIF-1α IN ARTICULAR CARTILAGE

In order to verify the results obtained with the chondrocyte cell cultures, we next performed immunofluorescence analysis for HIF-1α expression on frozen sections of OA and normal cartilage. The sections were probed with a...
rabbit polyclonal anti-HIF-1α antibody and the presence of HIF-1α was revealed with anti-rabbit IgG-biotin and streptavidin-Texas-red by confocal microscopy. Fig. 4 shows that HIF-1α could be readily detected in the nuclei of chondrocytes within the normal cartilage sections. A similar result was obtained when fresh frozen sections of OA human adult articular cartilage were probed with anti-HIF-1α antibody (Fig. 5).

**REGULATION OF HIF-1α IN OA AND NORMAL CHONDROCYTES BY TNF-α**

Pro-inflammatory cytokines, such as TNF-α, play a major role in the pathogenesis of degenerative joint disease. TNF-α has been implicated in the regulation of HIF-1α in other cell types and has been shown to modulate HIF-1α levels in a non-hypoxia dependent manner. Therefore, we decided to investigate whether TNF-α treatment of human articular chondrocytes had any effect on the levels of HIF-1α under normoxic conditions. Fig. 6 shows that treatment of normal or OA chondrocytes with 10 ng/mL TNF-α for 4 h resulted in an increase in the steady-state levels of HIF-1α. The increase in HIF-1α levels upon treatment with TNF-α was partially abrogated by pretreatment of the cells with either the p38 kinase inhibitor SB203580 or the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). We conclude, therefore, that TNF-α regulates HIF-1α levels in chondrocytes, at least in part, through NF-κB and p38 MAP kinase mediated pathways.

**Discussion**

The results presented in this study demonstrate for the first time the expression of HIF-1α and HIF-2α in human adult articular cartilage. The most important and provocative finding was that the expression of HIF-1α by cultured human articular chondrocytes could be readily detected under normoxic conditions. Furthermore, human articular chondrocytes that were grown in suspension culture under ambient O2 levels (21%) to preserve the differentiated phenotype exhibited an easily detectable level of HIF-1α protein expression as determined by immunoblotting, whereas chondrocytes that had become de-differentiated by culture in monolayer did not exhibit detectable HIF-1α expression. When cultured human OA and normal articular chondrocytes were exposed to hypoxia (1% O2) for up to 16 h, a modest increase in HIF-1α levels was observed. In some cases we observed a closely-spaced set of HIF-1α bands by immunoblotting. A closely spaced set of bands for HIF-1α has been observed by others and has been shown to be caused, at least in part, by phosphorylation of HIF-1α through the activity of the extracellular signal response kinases (ERKs). Interestingly, the presence of the lower molecular weight HIF-1α bands was mostly abrogated in articular chondrocytes in response to increasing duration of exposure to hypoxia. In other cell types hypoxia has been shown to induce the activity of ERKs. It remains to be determined whether the increase in the higher molecular weight HIF-1α bands upon
hypoxic treatment of articular chondrocytes was the result of increased phosphorylation, decreased degradation, or both. We also demonstrated that treatment of OA and normal articular chondrocytes with TNFα resulted in an increase in the steady state levels of HIF-1α. Pretreatment of the chondrocyte cultures with inhibitors of p38 MAP kinase or NF-κB partially abrogated the increase in HIF-1α levels in response to TNFα.

Articular cartilage, an avascular tissue, derives its oxygen and nutrient supply from the synovial fluid and to some extent from the underlying subchondral bone, with some of the chondrocytes in the deepest layers being up to 3–5 mm

Fig. 4. Localization of HIF-1α in normal cartilage by confocal microscopy. Frozen, fixed, permeabilized and blocked sections of normal articular cartilage slices were probed with anti-HIF-1α antibodies. HIF-1α localization was revealed with a biotinylated goat anti-rabbit IgG and streptavidin-Texas red. (A) Alcian blue staining of a serial section (400x). (B) Left panel: Image of lacuna. Right panel: Control section probed with the biotinylated goat anti-rabbit IgG and streptavidin-Texas red only (600x). (C) Left panel: Image of lacuna. Right panel: Frozen section probed with anti-HIF-1α antibody (600x). Verification of nuclear staining was achieved by counterstaining with DAPI (not shown).

Fig. 5. Localization of HIF-1α in OA cartilage by confocal microscopy. Frozen, fixed, permeabilized and blocked sections of OA cartilage slices were probed with anti-HIF-1α antibodies. HIF-1α localization was revealed with a biotinylated goat anti-rabbit IgG and streptavidin-Texas red. (A) Alcian blue staining of a serial section (400x). (B) Left panel: Image of lacuna. Right panel: Control section probed with the biotinylated goat anti-rabbit IgG and streptavidin-Texas red only (600x). (C) Left panel: Image of lacuna. Right panel: Frozen section probed with anti-HIF-1α antibody (600x). Verification of nuclear staining was achieved by counterstaining with DAPI (not shown).
Fig. 6. Stimulation of HIF-1α protein levels by TNF-α treatment. (A) Normal articular chondrocytes in suspension culture were either not treated, treated with 10 ng/ml TNF-α for 4 h or pre-treated with 10 µM of the p38 kinase inhibitor SB203580 for 1 h followed by treatment with 10 ng/ml TNF-α for 4 h. Nuclear extracts were then prepared and Western blot analysis performed with anti-HIF-1α antibodies. (B) OA chondrocytes in suspension culture were either not treated, treated with 10 ng/ml TNF-α for 4 h, pre-treated with 10 µM of the p38 kinase inhibitor SB203580 for 1 h followed by treatment with 10 ng/ml TNF-α for 4 h or pretreated with 20 µM of the NF-κB inhibitor PDTC for 1 h followed by treatment with 10 ng/ml TNF-α for 4 h. Nuclear extracts were then prepared and Western analysis performed with anti-HIF-1α antibodies.
from a supply artery. This has led to the assumption that articular cartilage is normally in a hypoxic environment as compared to vascularized tissues. Measurement of O₂ concentrations in synovial fluid and joint tissues along with in vitro studies support this view. We observed HIF-1α expression in chondrocytes cultured in suspension and in sections of articular cartilage but not in chondrocytes that had been de-differentiated by monolayer culture conditions. HIF-1α protein expression therefore correlates with the differentiated chondrocyte phenotype and our results lend support to the notion that cartilage is generally a hypoxic tissue. The aggregates and nodules of cartilage-like tissue that form in the suspension culture system utilized in the present work may result in a hypoxic environment thereby triggering the expression of HIF-1α in vitro. Interestingly, HIF-1α has been found to play a role in chondrocyte function and survival in the endochondral growth plate in vivo. When a targeted deletion of the HIF-1α gene in the cartilaginous growth plate of mice was performed, the chondrocytes within the interior of the growth plate, which are presumed to be hypoxic, underwent cell death. Furthermore, HIF-1α was found to be essential for the expression of the glycolytic enzyme phosphoglycerate kinase-1 in growth plate chondrocytes, indicating that a decrease in glycolytic activity may have contributed to the observed cell death. A similar role for HIF-1α in articular chondrocyte metabolism remains to be established.

Recent investigations have revealed that HIF-1α is regulated by factors other than hypoxia. Of pertinent interest to the field of chondrocyte biology and arthritis are the findings that HIF-1α levels and HIF-1 DNA-binding activity can be modulated by pro-inflammatory cytokines such as IL-1 and TNF-α, and also by growth factors and hormones such as TGF-β, IGF-1 and –2 and insulin. Indeed, HIF-1α was shown to be involved in the induction of insulin and IGF-responsive genes including glucose transporter and glycolytic enzyme genes, and VEGF. Even more recent work has elucidated that the activation of HIF-1α by insulin and insulin-like growth factors proceeds through a pathway dependent upon PI-3 kinase and MAP kinase in retinal epithelial, HepG2 and colon carcinoma cells, a regulatory mechanism distinct from that of hypoxia. However, in other work, insulin and IGF-1 stabilized HIF-1α in L8 myoblasts in a manner similar to hypoxic regulation. Moreover, IGF-2 and IGF-binding proteins 2 and 3 are HIF-1 target genes implicating HIF-1 in an IGF stimulated autocrine loop. Although the mechanism of HIF-1 activity by insulin and IGFs may be different in different cell types, the implications for articular chondrocytes are important, especially since IGF-1 and-2 are considered to be autocrine survival factors for these cells. Interestingly, the work herein and other investigations into the regulation of HIF-1α by cytokines such as IL-1 and TNF-α has revealed that they also cause an induction of HIF-1α in chondrocytes by cytokines such as TNF-α is, at least in part, modulated through NF-κB, induction of HIF-1α in NIH3T3 fibroblasts has also been reported to be mediated through activation of NF-κB. Similarly, p38 has been implicated in the induction of HIF-1α expression by thrombin in human vascular smooth muscle cells.

We also demonstrated the expression of HIF-2α mRNA in both normal and OA human articular chondrocytes. HIF-2α was originally described as an endothelial cell specific factor, however, more recent results indicate a more widespread expression pattern. HIF-2α can activate some of the same genes as HIF-1α, including erythropoietin and VEGF, and recent work indeed indicates that HIF-2α can activate a subset of the genes inducible by HIF-1α. Interestingly, this subset does not include the glycolytic genes that can be induced by hypoxia through HIF-1α. The functional significance of the HIF-2α mRNA expression in human articular chondrocytes observed in this work is currently being investigated.

It is believed that chondrocytes are the source of TNF-α expression found in OA and that chondrocytes respond to TNF-α in a catabolic fashion including the production of other inflammatory cytokines such as IL-1 and IL-8. Modulation of the metabolic status of chondrocytes in response to these cytokines is well established and it is interesting to speculate that some of this modulation occurs through a HIF-1 mediated pathway. In support of this notion, it has been reported that both IL-1 and TNF-α increase glucose transport in human articular chondrocytes and up-regulate mRNA expression of glucose transporters, which are HIF-1 target genes. We are currently trying to determine the subset of genes that are activated through HIF-1 by pro-inflammatory cytokines in articular chondrocytes. In conclusion, since HIF-1 modulates the activity of a large number of genes relevant to chondrocyte metabolism, growth, survival, and differentiation, the findings in this work concerning the expression of HIF-1α in articular chondrocytes and its induction by cytokines have important implications for its role in the regulation of the chondrocyte-specific phenotype in both normal and pathological situations.

Acknowledgements

The authors are grateful to Dr. Irving Shapiro and Dr. Chris Adams for use of their confocal microscope and to the NIH/NIAMS for grant support (Program Project AR-39740 to S.A.J.).

References

genes through the hypoxia-inducible factor HIF-1alpha/ARNT. EMBO J 1998;17:5085–94.


