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Hypoxia and HIF-1 α in osteoarthritis

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Abstract We have previously shown that functional inactivation of hypoxia-inducible factor-1 α (HIF-1 α) in growth-plate chondrocytes will dramatically inhibit anaerobic energy generation and matrix synthesis. Using immunohistochemistry, we have now analyzed the spatial distribution of HIF-1 α and its target genes in normal cartilage and in cartilage from knee joints with osteoarthritis. We detected HIF-1 α and its target genes in both types of cartilage. In cartilage from joints with osteoarthritis, the number of HIF-1 α -, Glut-1-, and PGK-1-stained chondrocytes increased with the severity of osteoarthritis. Activated matrix synthesis and strongly decreased oxygen levels are hallmarks of osteoarthritic cartilage. Thus, we assume that chondrocytes are depending on the adaptive functions of HIF-1 α in order to maintain ATP levels and thereby matrix synthesis during the course of osteoarthritis.

Résumé Nous avons montré précédemment que l'inactivation du facteur de l'hypoxie 1- α (HIF-1 α) dans les chondrocytes du cartilage de conjugaison inhibe très nettement la génération d'énergie anaérobie et la synthèse de la matrice. Utilisant l'immuno-histochimie nous avons analysé la distribution spatiale de HIF-1, et ses gènes-cibles dans le cartilage normal et dans le cartilage d'articulations avec arthrose. Nous avons détecté des HIF-1 α et ses gènes-cibles dans les deux types de cartilage. Dans le cartilage arthrosique le nombre de chondrocytes marqués HIF-1 α , Glut-1 et PGK-1 a augmenté avec la sévérité de l'arthrose. La synthèse de la matrice activée et le niveau d'oxygène

fortement diminué sont des caractéristiques du cartilage arthrosique. Donc nous supposons que les chondrocytes dépendent de la fonction adaptative de HIF-1 pour maintenir le niveau d'ATP et de cette façon la synthèse de la matrice pendant l'évolution de l'arthrose.

Introduction

Osteoarthritis (OA) is characterized by degeneration of articular cartilage, which is an avascular tissue getting its oxygen and nutrients from the dynamic flow of synovial fluid during joint movement [29]. Two independent groups have shown that the partial pressure of oxygen in synovial fluid obtained from normal joints ranges from 50 mmHg to 60 mmHg [10]. Silver et al. have further demonstrated that oxygen levels vary from 7% to 10% in the superficial zone to values as low as 1% in the deep zone of healthy cartilage [25]. In addition, Schneider et al. [22] have provided experimental evidence by using in vivo measurement techniques that oxygen tensions are further decreased in osteoarthritic joints. Based on these findings it is reasonable to assume an additional depletion of oxygen in osteoarthritic cartilage. We therefore hypothesized that survival and proper function of articular and especially OA, chondrocytes depend crucially on their ability to adapt to hostile microenvironmental conditions, most importantly characterized by extremely low oxygen levels.

Most mammalian cells possess an oxygen-sensing system and control oxygen homeostasis by up-regulation of a diverse set of genes including erythropoietin (EPO), vascular endothelial growth factor A (VEGF-A), inducible nitric oxide synthetase (iNOS), glucose transporters, enzymes of the glycolytic pathway, IGF-binding-protein-3 (IGFBP-3) and leptin. This response to hypoxia is controlled and organized most prominently by hypoxia-inducible factor-1 (HIF-1), which belongs to the family of basic helix-loop-helix (bHLH)-containing PER-ARNT-SIM (PAS) domain transcription factors. HIF-1 is a heterodimer consisting of HIF-1 β and HIF-1 α subunits [24]. It has been repeatedly shown that HIF-1 is of fundamental

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importance in tumor genesis, inflammation, ischemic tissues and growth-plate development [3, 21, 23]. Two independent studies have demonstrated HIF-1 mRNA expression by articular chondrocytes in vivo [1, 26]. The active subunit α of HIF-1 confers the oxygen responsive-

ness and is hydroxylated by a set of oxygen-sensitive prolyl-hydroxylases under normoxic conditions, a process that initiates its degradation through the proteasome after coupling to ubiquitin [10]. In addition, recent studies have shown that mechanical stress and proinflammatory cy-

Fig. 1 Evidence for an accumulation and increased transcriptional activity of HIF-1 α in OA cartilage. **A** Safranin-O staining of normal articular cartilage (Mankin-grade 0). **B** Immunohistochemical detection of hypoxia-inducible factor-1 α (HIF-1 α) in normal cartilage (arrowheads indicating positive brown chondrocytes). **C** Displays an immunohistochemical staining of Glut-1 in normal cartilage (arrowheads indicating positive red chondrocytes). **D** Demonstrates an immunohistochemical staining of PGK-1 in healthy articular cartilage (arrowheads indicating positive red chondrocytes). **E** Safranin-O staining of severely affected OA cartilage (Mankin grade 12). **F** Immunohistochemical detection of HIF-1 α in severe OA cartilage (arrowheads indicating positive brown chondrocytes). **G** Glut-1 is strongly expressed in severe OA cartilage specimens (arrowheads

indicating positive red chondrocytes). **H** Even PGK-1 is strongly detectable in severe OA cartilage specimens (arrowheads indicating positive red chondrocytes). **I** High-power magnification of HIF-1 α -positive chondrocytes in the deep zone of healthy articular cartilage (arrowheads indicating positive brown chondrocytes). **J** Mild OA cartilage showing a high number of HIF-1 α -stained chondrocytes in the middle zone. **K** High-power magnification of HIF-1 α -positive chondrocytes within a chondrocyte cluster next to the surface of a severely affected sample (arrowheads indicating positive brown chondrocytes). **L** PGK-1 immunostaining showing PGK-1-positive erythrocytes and chondrocytes in a vessel crossing the tidemark (arrowheads indicating a positive red chondrocyte and erythrocytes).

tokines, e.g., interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), are able to stabilize HIF-1 α thereby up-regulating the transcription of HIF-1 target genes [2, 9]. In this context, it is of special interest that both mechanical stress and pro-inflammatory cytokines are considered to play an important role in the pathogenesis of OA.

Although many target genes of HIF-1 are expressed in healthy and OA cartilage, thus far, no data have been published about HIF-1 expression patterns in this setting. Interestingly, many HIF-1 target genes expressed in articular cartilage are up-regulated through OA progression, for example, VEGF-A, IGFBP-3, and iNOS [7, 12, 16, 27]. In addition, Dumond et al. [4] reported increased leptin levels in cartilage specimens and synovial fluids from OA joints. Leptin is a small polypeptide mainly produced by adipocytes and encoded by the obese (*ob*) gene with systemic effects on energy expenditure and food intake. Interestingly, previous studies on the regulation of the *ob* gene have shown that leptin synthesis is significantly increased by hypoxia in an HIF-1-dependent manner [5, 6].

In the present paper, we characterize the spatial protein expression pattern of HIF-1 α during the progression of OA. Functional activity of the HIF-1 transcription factor was indirectly assessed by immunohistochemistry with monospecific antibodies against the HIF-1 target genes glucose transporter-1 (Glut-1) and phosphoglycerate kinase-1 (PGK-1) in normal and OA cartilage.

Materials and methods

Five normal and 24 OA cartilage/bone samples were obtained during total knee replacements or autopsies. Data were carefully reviewed to exclude any secondary forms of OA and rheumatoid arthritis. Specimens were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline, decalcified in 0.2 M EDTA, embedded in paraffin wax and then cut into 6- μ m-thick sections perpendicular to the surface. Routinely, safranin-O-stained sections of normal and osteoarthritic cartilage specimens were graded according to Mankin by two different investigators [11]. Furthermore, the samples were classified into normal, mild, moderate, or severe osteoarthritic lesions, as described previously [13]. For immunohistochemical analyses, three primary antibodies against HIF-1 α (monoclonal, Novus Biologicals, 1:10,000) and Glut-1 (polyclonal, Santa Cruz Biotechnology, 1:50) and PGK-1 (polyclonal, Santa Cruz Biotechnology, 1:100) were used (overnight at 4°C). For visualization of HIF-1 α , a catalyzed signal amplification kit (CSA Kit, DakoCytomation) based on a streptavidin–biotin peroxidase reaction was employed and diaminobenzidine (DAB) was used as a chromogen. For Glut-1, sections were pretreated with Tris–HCl (pH 10) at 95°C and trypsin (1 mg/ml) at 37°C. No pretreatment was used for PGK-1. Control sections were incubated with nonimmune rabbit or goat antisera. Primary antibodies were followed by incubation with biotinylated antirabbit or anti-goat antibodies. For Glut-

1 and PGK-1, a complex of streptavidin and biotin labelled with alkaline phosphatase was added according to the protocol of the manufacturer. Finally, the sections were stained with Fast Red and counterstained with hematoxylin.

Results

Hypoxia-inducible factor-1 α and its targets Glut-1 and PGK-1 were detected by immunohistochemistry in normal and OA cartilage (Fig. 1A). In healthy articular cartilage, HIF-1 α was mainly undetectable. In a few samples, single chondrocytes stained positive for HIF-1 α (Fig. 1B). HIF-1 target genes Glut-1- and PGK-1-stained chondrocytes were commonly seen in the middle and deep zone of normal cartilage (Fig. 1C,D). The overall numbers of HIF-1 α -, Glut-1-, and PGK-1-positive chondrocytes increased in parallel to the progressive cartilage damage (Fig. 1E–H). In OA samples specifically, hypertrophic chondrocytes displayed a strong staining of HIF-1 α (not shown). These chondrocytes are considered to produce type X collagen, osteopontin and annexins, probably mineralizing their surrounding matrix [17, 19, 30]. In OA cartilage, the transcription factor HIF-1 α was mostly expressed in the same chondrocytes showing Glut-1 and PGK-1 staining. However, in some samples with high numbers of PGK-1- and Glut-1-positive chondrocytes, the transcription factor was undetectable. This phenomenon might result from the rapid degradation of HIF-1 α after exposure to 21% oxygen (during total knee replacement). The highest numbers of chondrocytes positive for HIF-1 α and its target genes Glut-1 and PGK-1 were found in severely affected specimens (Fig. 1E–H, K). Control sections displayed no staining (not shown).

Discussion

In summary, our study on the expression patterns of HIF-1 α and its target genes Glut-1 and PGK-1 in normal and OA cartilage suggests that HIF-1 α transcriptional activity increases during the progression of OA. A recent study by Richardson et al. [20] described the expression of Glut-1 and 3, both target genes of HIF-1 α , in normal articular chondrocytes and proposed a critical role of glucose uptake in articular cartilage metabolism. Our data confirm these observations and further suggest that increased synthesis of glucose transporter and glycolytic enzymes might result from accumulated HIF-1 α , possibly caused by reduced oxygen levels during the progression of OA. This hypothesis is further strengthened by previous studies from our and other groups demonstrating increased VEGF-A synthesis in late stages of OA [16, 18]. Beside hypoxia, increased levels of IL-1 β and mechanical forces are reasonable candidates as inducers of the transcription factor HIF-1 α in OA cartilage. In epiphyseal chondrocyte cultures, where we functionally inactivated HIF-1 α by using the Cre/loxP method, we have shown that HIF-1 α is of pivotal importance for ATP generation under aerobic

and anaerobic conditions by regulating glucose uptake as well as activity of key glycolytic enzymes [14]. Even chondrocytic matrix synthesis is significantly influenced by HIF-1 α , possibly through its control over energy generation [14]. Furthermore, it has been repeatedly reported that OA chondrocytes display a fundamental metabolic activation, resulting in increased mRNA levels of extracellular matrix components and several other molecules [1, 28]. Thus, in view of the decreased oxygen levels in OA synovial fluids and the dramatic inhibiting effects of functional inactivation of HIF-1 α for chondrocytic energy generation and matrix synthesis, it is reasonable to assume that HIF-1 possesses an important role for cartilage biology and for the pathogenesis of OA. In addition, it seems likely that activated chondrocytes rely on HIF-1 to increase their anaerobic energy generation in order to compensate for the strongly increased ATP consumption in OA. We conclude that HIF-1 is a critical factor for OA chondrocytes to survive the harsh microenvironmental conditions similar to growth-plate chondrocytes [15, 21]. Further studies are needed to clarify the detailed role of HIF-1 α in articular cartilage and whether HIF-1 is the key player for maintenance of energy generation during OA.

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