O-linked N-acetylglucosamine (O-GlcNAc) protein modification is increased in the cartilage of patients with knee osteoarthritis

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SUMMARY

Objective: There is increasing evidence that the addition of O-linked N-acetylglucosamine (O-GlcNAc) to proteins plays an important role in cell signaling pathways. In chondrocytes, accumulation of O-GlcNAc-modified proteins induces hypertrophic differentiation. Osteoarthritis (OA) is characterized by cartilage degradation, and hypertrophic-like changes in hyaline chondrocytes. However, the mechanisms responsible for these changes have not been described. Our aim was to study whether O-GlcNAcylation and the enzymes responsible for this modification are dysregulated in the cartilage of patients with knee OA and whether interleukin-1 could induce these modifications in cultured human OA chondrocytes (HOC).

Design: Human cartilage was obtained from patients with knee OA and from age and sex-matched healthy donors. HOC were cultured and stimulated with the catabolic cytokine IL-1α. Global protein O-GlcNAcylation and the synthesis of the key enzymes responsible for this modification, O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), were assessed by western blot.

Results: OA was associated with a 4-fold increase in the global O-GlcNAcylation in the cartilage. OA cartilage showed a re-distribution of the OGT and OGA isoforms, with a net increase in the presence of both enzymes, in comparison to healthy cartilage. In HOC, IL-1α stimulation rapidly increased O-GlcNAcylation and OGT and OGA synthesis.

Conclusions: Our results indicate that a proinflammatory milieu could favor the accumulation of O-GlcNAcylated proteins in OA cartilage, together with the dysregulation of the enzymes responsible for this modification. The increase in O-GlcNAcylation could be responsible, at least partially, for the re-expression of hypertrophic differentiation markers that have been observed in OA.

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Introduction

Osteoarthritis (OA) is recognized as a whole organ disease mainly characterized by the gradual loss of articular cartilage. Although alternative routes have been proposed, research has focused on the change in chondrocyte metabolism that leads to cartilage degradation. It has been proposed that during OA, articular chondrocytes lose their quiescent surrounding, characterized by type II collagen (collII) and aggrecan, and regain a behavior with similarities to hypertrophic chondrocytes in the growth plate of growing individuals, although this is controversial. Several studies have demonstrated that different markers of chondrocyte hypertrophy, such as colX, matrix metalloproteinase 13 (MMP13), Indian Hedgehog (IHH), Runx-2, osteocalcin, osteopontin or alkaline phosphatase (ALP), are increased in OA cartilage, at least in certain subsets of patients, or in different stages of the disease. The ultimate end stage of chondrocyte hypertrophy in the growth plate is calcium deposition, allowing bone to enlarge. Although calcification does not have to be unavoidably reached in OA cartilage, tidemark duplication and an increase in calcified cartilage thickness are frequently observed.

The addition of the single-sugar N-acetylgalactosamine (O-GlcNAc) by O-linkage (O-linked β-N-acetyl-glycosylation, O-GlcNAcylation) to nuclear, mitochondrial and cytoplasmic proteins is a form of protein glycosylation that has emerged as an important regulatory mechanism for numerous cellular processes. Over
three thousand nuclear and cytoplasmic proteins, belonging to almost every functional class of proteins, including transcription factors, cytoskeletal proteins, RNA polymerases, cell cycle regulators, phosphatases and kinases are known to be O-GlcNAcylated\textsuperscript{3,4}. Analogous to phosphorylation, this dynamic and reversible process of post-translational modification is responsive to a great variety of stimuli, including stress and nutrient metabolism\textsuperscript{3-5}. The attachment of the sugar is controlled by two highly conserved enzymes, the O-GlcNAc transferase (OGT) and by the neutral N-acetylglucosaminidase O-GlcNAcase (OGA), that catalyzes the hydrolytic cleavage of O-GlcNAc form proteins\textsuperscript{4,5}. The donor substrate for the reaction, UDP-GlcNAc, is synthesized by the hexosamine biosynthesis pathway (HBP), a major branch of the glycolytic pathway that metabolizes 2\textsuperscript{-}3-ketoisocaproate (KIC), a major branch of the glycolytic pathway that metabolizes 2\textsuperscript{-}3-ketoisocaproate (KIC).

The alteration in protein O-GlcNAcylation has been described to have a pathogenic role in various chronic and age related human diseases including diabetes mellitus, cardiovascular disease, neurodegeneration and cancer\textsuperscript{4,6}. Despite the importance described for O-GlcNAcylation in degenerative and age related diseases, its role in OA is completely unknown. We have recently reported that O-GlcNAcylation is increased during the hypertrophic differentiation of chondrocytes\textsuperscript{7}. Furthermore, the accumulation of O-GlcNAcylated proteins per se was able to induce a significant expansion in the growth plate height in newborn mice, and the hypertrophic differentiation of chondrocytes, increasing the gene expression of coI\textsubscript{X}, Ihh, Alp, and Runx-2\textsuperscript{5}. These markers are also increased in human OA cartilage, although the mechanisms associated to the induction of this phenotype have not been clearly established.

The concentration of the proinflammatory and catabolic factor interleukin-1\textsubscript{x} (IL-1\textsubscript{x}) is increased in OA joints. Furthermore, IL-1\textsubscript{x} is able to induce an increase in the matrix degrading enzymes found in the OA joint. Therefore, the aim of this work was to analyze whether O-GlcNAcylation and the presence of the different isoforms of OGT and OGA, are modified in the articular cartilage from patients with knee OA. We also studied whether the presence of IL-1\textsubscript{x} is able to induce O-GlcNAcylation or to modify the presence of the regulating enzymes, in cultured human OA chondrocytes (HOC).

Materials and methods

**Human articular cartilage samples**

OA knee articular cartilage was obtained during the replacement surgery (Orthopaedic Surgery Dept. Fundación Jiménez Díaz, Madrid, Spain). Twelve OA patients were included in this study (six women/six men; mean age 71 ± 3 years; Mankin score between 11 and 14). Healthy human knee cartilage was obtained from ten donors (four women/four men; mean age: 65 ± 3 years; Mankin score between 1 and 4) without a known history of joint disease (Transplant Services Foundation, Hospital Clinic, Barcelona, Spain). Informed consent was obtained from all individuals. The Medical Ethics Committee of the Fundación Jiménez Díaz and Hospital Clinic approved the protocol.

HOC for primary culture were isolated from the OA cartilage by sequential digestion with pronase and collagenase. Chondrocyte culture was characterized by safranin O and alcan-blue staining and gene expression (type II and X collagen, aggrecan) (data not shown). Chondrocytes were grown to confluence in Dulbecco’s Modified Eagle’s Medium (DMEM) (4.5 g/L glucose; Lonza; Verviers, Belgium) supplemented with 10% fetal calf serum (Lonza), 60 U/ml penicillin, 60 μg/ml streptomycin and 2 mmol/L glutamine (Lonza) at 37°C in the presence of 5% CO\textsubscript{2}. Experiments were performed with first or second passage cells. In each experiment, cells were made quiescent for 48 h in DMEM medium without serum and stimulated with 10 ng/ml IL-1\textsubscript{z} (Sigma Aldrich, St Louis, MO, USA) for different periods of time.

**Western blot**

Proteins from human cartilage or HOC were isolated in ice-cold lysis buffer [15 mM Hepes pH 7.4; 250 mM NaCl; 10% glycerol; 0.5% NP-40; 0.5 mM Ethylene-diamine-tetracetic acid (EDTA)] in the presence of the specific protease inhibitors PMSF 0.1 mM (Phenyl--methyl--sulfonyl--fluoride) and Protease Inhibitor Cocktail (both from Sigma). The lysates were centrifuged at 12,000 × g for 10 min twice. Protein concentrations were determined by a colorimetric method. Equal amounts of proteins were resolved on 8% acrylamide—sodium dodecyl sulphate (SDS) gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Western blots were carried out as previously described employing antibodies against O-GlcNAc-modified proteins (RL2: Thermo Fisher Scientific, Waltham, CA, USA), OGT (DM17: Sigma, St. Louis, MO, USA) and OGA 345 (kindly donated by Dr GW Hart, Johns Hopkins University School of Medicine, Baltimore, USA)\textsuperscript{6}. Bands were quantified by densitometry and results expressed as arbitrary units (Quantity One Software 4.6.3). For total O-GlcNAc quantification, all the immunoreactive bands were merged\textsuperscript{6}.

**Statistical analysis**

Results are expressed as mean values with 95% confidence intervals. Comparisons between multiple groups were performed using Kruskal–Wallis test and comparison of two groups were analyzed using the Mann–Whitney U test. A P value of less than 0.05 was considered statistically significant.

**Results**

**Quantification of O-GlcNAcylated proteins in the cartilage**

As can be observed in Fig. 1(A), OA cartilage showed around a 4-fold increase in the amount of O-GlcNAcylated proteins in comparison to healthy cartilage, with the most extensive O-GlcNAcylation observed in the areas of 45–70 and 120–170 kDa. There was little O-GlcNAcylation of proteins of less than 40 kDa in size, or higher than 170 kDa.

**Synthesis of OGT and OGA in the human cartilage**

In the human cartilage we identify all the three different OGT isoforms that have been previously described as ncOGT (nuclear and cytoplasmic OGT), mOGT (mitochondrial OGT) and sOGT (short OGT). While ncOGT and mOGT were downregulated, we observed a marked increase in sOGT in OA patients in comparison to healthy cartilage [Fig. 1(B)]. Regarding OGA, both isoforms described in humans were identified in the cartilage\textsuperscript{3,4}. While the synthesis of OGA-L, the full-length isoform, was found to be decreased in the OA cartilage, there was a clear increase in the OGA-S isoform in the OA cartilage in comparison to healthy one [Fig. 1(B)].

**Alterations in the amount of O-GlcNAcylated proteins following IL-1 stimulation in HOC**

To gain insight into the relationship between O-GlcNAcylation and OA associated events in the cartilage, we measured the effect of the catabolic cytokine IL-1\textsubscript{z} on HOC in culture. IL-1\textsubscript{z} treatment resulted in an accumulation of O-GlcNAcylated proteins in HOC in a time-dependent manner, with a peak of induction 6 h after...
stimulation [Fig. 2(A)]. Regarding OGT, IL-1α increased the presence of all three OGT isoforms as early as 3 h, although the peak of induction was at 6 h. IL-1α also increased the presence of OGA-S isoforms after 3 and 6 h of stimuli [Fig. 2(B)].

Discussion

O-GlcNAcylation has been described as a key regulator of cell/tissue function. Thousands of proteins, such as chromatin-associated proteins, transcription factors, proteasomal proteins, cytoskeletal proteins, kinases or metabolic enzymes, are O-GlcNAcylated. Here, we have described that in human OA cartilage there is an accumulation of O-GlcNAcylated proteins associated to an alteration in the expression of the enzymes that regulate this glycosylation. To our knowledge, this is the first description of an activation of protein O-GlcNAcylation in the cartilage associated to a human joint pathology. However, we recently described that O-GlcNAcylation has a chondromodulating activity because it was able to increase the expression of hypertrophic differentiation markers in chondrocytes. Pathological chondrocyte hypertrophy is observed in OA associated with the re-emergence of a “developmental” pattern of gene and protein expression in the cartilage. Interestingly, we observed that protein O-GlcNAcylation accumulation in cultured chondrocytes preceded the increase in the gene expression of hypertrophic markers. Furthermore, O-GlcNAcylation per se was able to induce chondrocyte hypertrophy. So, the increased O-GlcNAcylation observed in the cartilage of OA patients could account, at least partially, for the increased expression of hypertrophic markers that have been described in OA cartilage. Chronic increases in O-GlcNAcylation have also been associated to cell hypertrophy in renal and cardiac cells, that is characterized by cell cycle arrest and the inhibition of cell proliferation. An increase in local O-GlcNAcylation has been described during human cardiac hypertrophy, together with a dysregulation of OGT and OGA.

Fig. 1. O-GlcNAcylated protein levels and OGT and OGA presence in human healthy (white dots) and OA (black dots) knee cartilage. (A) Representative western blot and densitometric analysis for O-GlcNAcylated protein measurement using RL2 antibody. (B) Representative western blot and densitometric analysis for the three OGT isoforms, nucleo-cytoplasmic (nOGT), mitochondrial (mOGT) and small (sOGT); and the two OGA isoforms, long (OGA-L) and short (OGA-S). EZ blue staining was used as protein loading controls. Results are expressed as fold induction for each patient, and the mean ± 95% C.I. n = 10 healthy and n = 12 OA subjects; P values calculated by Mann–Whitney test.
in the heart. So, the reactivation of fetal gene program associated with cardiomyocyte hypertrophy was also concomitant with an increase in O-GlcNAcylation. Regarding kidney cells, the hypertrophy of mesangial cells was associated to an increase in protein O-GlcNAcylation both in patients and in cells in culture. A chronic dysregulation in the level of protein O-GlcNAcylation has been described in different chronic human pathologies, including diabetes, cardiomyopathy, vascular dysfunction, diabetic nephropathy or cancer. The contribution of this type of glycosylation to the etiology of these diseases has been especially underlined in diabetes and diabetic complications, where the increase in O-GlcNAcylation has been proposed, at least partially, as the cause for the toxic effects of glucose. Different genetic or enzymatic manipulations that increased O-GlcNAc were able to induce insulin resistance or hyperleptinemia, suggesting a direct role in the induction of the disease. In experimental models of diabetes, excessive O-GlcNAcylation preceded histologic and functional alterations in diabetic cardiomyopathy. Furthermore, reducing the level of O-GlcNAc, reversed some of the complications associated to this disease. So, a chronic increase in O-GlcNAcylation seems to be associated to the toxic effects of glucose or to an impairment in the management of nutrients/energy. OA has also been associated to a dysregulation in glucose and ATP metabolism in the chondrocyte. HOC showed an altered ability to regulate glucose entry in the cell, together with a reduced activity of the mitochondrial electron transport complexes. An increase in

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Fig. 2. (A) O-GlcNAcylated protein levels and (B) OGT and OGA presence in HOC stimulated with IL-1α for different periods up to 24 h. Representative western blot and densitometric analysis for O-GlcNAcylated protein detection using RL2 antibody the three OGT isoforms, nucleo-cytoplasmic (ncOGT), mitochondrial (mOGT) and small (sOGT), and the two OGA isoforms, long (OGA-L) and short (OGA-S). Results are shown as fold change vs 0 h ± 95% CI of four independent experiments. P values calculated by Mann–Whitney test vs Basal.
O-GlcNAcylation in OA cartilage could be probably responsible for the reduced activity of these mitochondrial complexes demonstrated in HOC. The increase in O-GlcNAcylation observed in OA cartilage was associated with a re-distribution of the expression of OGT isoforms, with a net increase in OGT. Increased expression of OGT has been associated with O-GlcNAc accumulation both in response to acute stress and chronic damage. We also observed an increase in OGA expression, the enzyme which removes O-GlcNAc, in the OA cartilage. In HOC, O-GlcNAc accumulation paralleled an increase in both OGT and OGA. Our results are consistent with recently published data, suggesting that OGT and OGA may increase or decrease in tandem in response to a modification in O-GlcNAc levels.1,6

We have also observed that IL-1, a key catabolic cytokine in OA, is able to induce O-GlcNAc accumulation in HOC. Recent data have also implicated global and specific sites of O-GlcNAcylation in the inflammatory response induced by IL-1β. Our data indicate that IL-1β is able to induce the modification of the O-GlcNAc system in the cartilage thus supporting the relationship between OA and O-GlcNAc levels.

In conclusion, these results demonstrate that there is a dysregulation of O-GlcNAcylation of cartilage proteins during human OA together with changes in the expression of OGT and OGA isoforms that could be probably induced by proinflammatory cytokines. O-GlcNAc accumulation could be responsible, at least partially, for the re-expression of hypertrophic differentiation markers that have been observed in OA cartilage. Our data would link OA pathophysiology to alterations in key glucose sensor pathways, and support the hypothesis that O-GlcNAcylation may play an important role in the development of chronic and age-related diseases.

Contributions

Conception and design: LT, JA-B; NEZ; GH-B and RL; Acquisition and assembly of data: LT, JA-B, NEZ and AL-V; Analysis and interpretation of the data: LT, JA-B; NEZ; AL-V; GH-B and RL; Drafting of the article: GH-B and RL; Critical revision of the article for important intellectual content: all authors; Final approval of the article: all authors; Provision of patients and controls: CR-V.

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Competing interests

Authors declare no conflict of interest.

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