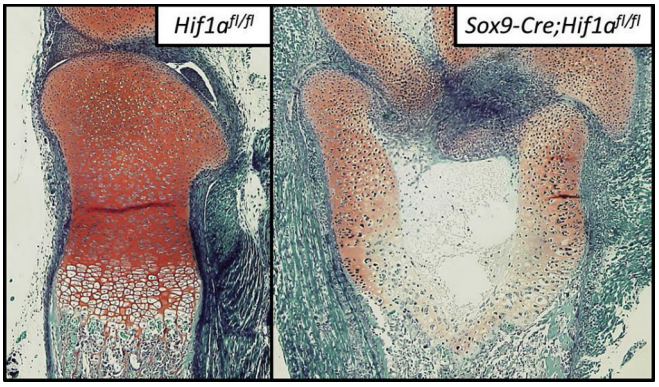
showed a decrease in proteoglycan content in comparison with the control Hif1afl/fl embryo. TUNEL staining accentuated ectopic apoptosis broadly across the limb cartilage, and immunofluorescence showed enhanced expression of Mmp13 surrounding the defective area. When we created the experimental OA model one week after the tamoxifen injection into Col2a1-CreERT2;Hif1afl/fl mice, the OA development in the knee joints was markedly accelerated as compared to the control Hif1afl/fl joints. Apoptosis and Mmp13 expression were upregulated by the conditional knockout of HIF-1α after maturation in articular cartilage, as well as in the limb cartilage. To reveal altered gene expression by HIF-1α deficiency, we obtained RNA samples directly from the limb cartilages of Sox9-Cre;Hif1afl/fl and the control Hif1afl/f embryo. Real-time RT-PCR using these samples revealed increases of catabolic factors including Mmp13 and Mmp9, and decreases of anabolic factors including Col2a1 and Sox9 by the conditional knockout of HIF-1α. When we deleted HIF-1α in primary articular chondrocytes from Hif1afl/fl mice by adenoviral vector expressing Cre recombinase, expressions of the catabolic and the anabolic genes were changed in ways similar to those in the in vivo analyses, under both normoxic and hypoxic (1% O2 concentration) conditions. Similar results were also obtained under both O2 conditions by HIF-1α silencing in primary articular chondrocytes from WT mice using siRNA. Furthermore, in the organ culture of mouse femoral heads, stabilizing HIF-1α protein by CoCl2 treatment markedly decreased aggrecan release into the medium.

Conclusions: HIF-1α regulates the configuration and maintenance of articular cartilage through induction of anabolic factors and suppression of catabolic factors. Elucidation of the molecular network related to HIF-1α may lead to cartilage regeneration and OA treatment.



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DNA REPAIR ENZYME, APURINIC/APYRIMIDINIC ENDONUCLEASE 2 (APEX2), HAS A POTENTIAL TO PROTECT AGAINST THE DOWN-REGULATION OF CONDROCYTE ACTIVITY IN OSTEOARTHRITIS

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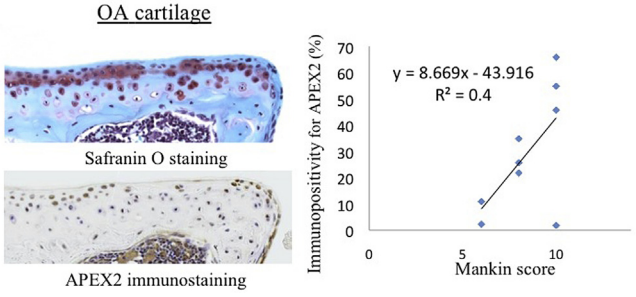
Background: Recent reports clearly indicate that chronic excess production of reactive oxygen species (ROS) from chondrocytes, which is induced by mechanical force to cartilage, plays an important role in cartilage degeneration occurring after mechanical injury to cartilage in osteoarthritis (OA). However, pathogenic mechanism of ROS-mediated degeneration of articular cartilage remains unknown. While studies have provided ample confirmation of the generation of ROS in OA cartilage, the activity of cellular antioxidants in degenerated articular cartilage still remains unclear.

Propose: Apurinic/apyrimidinic endonuclease 2 (Apex2) is an essential DNA repair enzyme that plays a critical role in DNA repair against the oxidative damage in a variety of human somatic cells. We postulated that Apex2 in chondrocytes may have a role to protect against the catabolic process of articular cartilage in OA. The aim of the study was to examine the potential involvement of DNA repair enzyme Apex2 in the pathogenesis of OA.

Methods: Expression of Apex2 was histologically investigated in OA articular cartilages from STR/OrtCrlj mice, an experimental animal model which spontaneously develops an osteoarthritic process. We examined whether OA-related catabolic factor [interleukin (IL)-1β] influenced the expressions of Apex2 in human chondrocytes. Knockdown of Apex2 with small interfering RNA (siRNA) was also performed to investigate whether Apex2 is associated with cellular activity and survival in human chondrocytes.

Results: In OA mouse chondrocytes, higher levels of Apex2 expressions were histologically observed in the severe OA cartilages than in mild degenerated cartilages. The immunopositivity of Apex2 was significantly correlated with the degree of cartilage degeneration (Figure). OA-related catabolic factor, IL-1β, induced the expression of Apex2 in chondrocytes. Apex2 silencing using siRNA reduced the chondrocyte activity in vitro.

Conclusions: The expression of DNA repair enzyme Apex2 in chondrocytes was associated with the degeneration of articular cartilages and was induced by OA-relating catabolic factor. Our findings suggest that Apex2 may have a potential to prevent the catabolic stress-mediated down-regulation of chondrocyte activity in OA.



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HYPERGLYCEMIA-LIKE CULTURE CONDITIONS INDUCE IL-1B AND TNF-α EXPRESSION AND IMPAIR AUTOPHAGY IN HUMAN CHONDROCYTES

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Purpose: Accumulating evidence indicates that Diabetes Mellitus (DM) is an independent risk factor for severe osteoarthritis (OA). Understanding the mechanisms involved is essential for designing preventive strategies and targeted therapies that can halt OA progression in DM patients. In this context, we hypothesized that hyperglycemia, a hallmark of DM and other conditions associated with glucose imbalance, is a major effector of chondrocyte damage. Accordingly, our previous studies showed that culture of human chondrocytes under excess glucose favors catabolic responses and oxidative stress. This study aims at further characterizing the effects of hyperglycemia in human chondrocytes by determining whether it also promotes inflammatory responses. Furthermore and to gain some insight as to the mechanisms by which hyperglycemia favors OA progression, modulation of autophagy was also evaluated as a crucial mechanism for the elimination of damaged proteins and organelles whose impairment has been implicated in the deleterious effects of hyperglycemia in various cells.

Methods: Articular cartilage was obtained from multi-organ donors (44-73 years old, mean = 59.4, n = 10) at the Bone Bank of the University and Hospital Center of Coimbra with approval by the Ethics Committee. Isolated chondrocytes and the human chondrocytic cell line, C28/I2 (kindly provided by Prof. Mary Goldring and Harvard University), were cultured in Ham-F12 or DMEM:Ham F-12 (1:1), respectively, containing regular (10 mM) or excess (30 mM) glucose for various periods. The expression of pro-inflammatory markers (IL-1β and TNF-α) was evaluated by qRT-PCR. Autophagy was assessed by determining the protein levels of LC3-I and II in the presence and absence of the lysosome inhibitor, chloroquine. To rule out possible osmotic effects, parallel experiments were performed in the presence of the cell-impermeable polyol, mannitol. Cell viability was evaluated by the MTT reduction assay.

* These authors contributed equally to this work

Results: Culture of human chondrocytes in high glucose (30 mM) for up to 48h had no effect on the viability of either primary chondrocytes or C28/I2 chondrocytic cells. Exposure to high glucose (30 mM) for 24h significantly increased IL-1β and TNF-α mRNA levels (196.8±33.6%, P = 0.0066 and 285.4±54.5%, P = 0.0045, respectively). On the other hand, LC3-II levels, as well as the ratio LC3-II/LC3-I, were significantly reduced by culture of C28/I2 cells in high glucose for 24h, remaining low at 48h, either in the presence or absence of chloroquine. This indicates that LC3-II synthesis was decreased relative to cells maintained in regular glucose medium. Culture in mannitol-containing medium (20 mM in medium containing 10 mM glucose) for up to 48h had no significant effect on LC3-I and II levels.
Conclusions: Hyperglycemia-like glucose concentrations are sufficient to induce inflammatory responses and impair autophagy in human chondrocytes which can contribute to the development and progression of OA in patients with DM and other conditions associated with impaired glucose homeostasis.
This work was supported by FEDER and the Portuguese Foundation for Science and Technology (FCT) grants PEst-C/SAU/LA0001/2011 and PTDC/EME TME/113039/2009 and PhD fellowships SFRH/BD/47470/2008 and SFRH/BD/78188/2011.

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MCPIP1 REGULATES THE EXPRESSION OF INTERLEUKIN-6 IN HUMAN OSTEOARTHRITIS CHONDROCYTES

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Purpose: Post-transcriptional regulation of cytokine expression is important s for maintaining tissue integrity. MCPIP1 (also known as ZC3H12A) was identified as a novel protein, which harbors CCCH-type zinc-finger domain and a PIN-like RNase domain. MCPIP1 destabilizes inflammatory cytokines mRNAs via their 3' UTR. Osteoarthritis (OA) affects 27 million Americans and is a leading cause of disability worldwide in elderly. IL-6 has recently gained attention because of its high levels in synovial fluid and ability to induce MMP-13 in OA. In the present study we determined whether MCPIP1 regulates IL-6 expression in human OA chondrocytes.
Methods Human cartilage samples were obtained from OA patients who underwent total joint arthroplasty and chondrocytes were prepared by the enzymatic digestion. For gene expression analysis total RNA was isolated from cultured primary chondrocytes or from damaged or smooth OA cartilage using RNeasy mini kit (Qiagen). RNA fluorescent in-situ hybridization (ISH) for IL-6 and MCPIP1 expression was performed using RNAScope (ACD, CA) according to the instructions. Wild type or mutant MCPIP1 was overexpressed in chondrocytes transfected with the cDNA constructs using Amaxa. Knockdown experiments were performed using Trisilencer-27 human siRNAs (Origene). For RNA immunoprecipitation, chondrocytes were stimulated with IL-1β (1ng/ml) for 12 h and then treated with 1% formaldehyde to cross link protein-RNA complexes. After sonication, extracts were centrifuged and lysates were incubated overnight with isotype control IgG or with anti-MCPIP1 antibody (Origene). After multiple stringent washes and reverse cross-linking RNA was purified using RNeasy mini kit. Expression of IL-6 and MCPIP1 was assessed using TaqMan assays (Applied Biosystems, Carlsbad, CA). Expression of IL-6 targeting miRNAs in chondrocytes was quantified by TaqMan assays.
Results: High levels of IL-6 mRNA were induced in chondrocytes stimulated with IL-1β in a time dependent manner and peaked at 8h post-stimulation and then declined. On the other hand, expression of MCPIP1 mRNA peaked at 6h post-stimulation with IL-1β. Using multiplex RNA fluorescent ISH, expression of both IL-6 and MCPIP1 mRNA in IL-1β stimulated human chondrocytes, also showed that IL-6 mRNA expression was relatively high compared to MCPIP1 mRNA expression with distinct speckle patterns. Both IL-6 and MCPIP1 mRNAs were localized in the nuclei as well as in the cytoplasm. Overexpression of wild type MCPIP1, but not mutant MCPIP1, reduced the expression of IL-6 mRNA. Importantly siRNA-mediated knockdown of MCPIP1 elevated the IL-6 mRNA expression in human chondrocytes. Interestingly, alteration in gene expression of other OA markers (MMP3, ACAN or COL10A1) was not observed, suggesting that these are not regulated by MCPIP1. To confirm the binding of MCPIP1 with IL-6 mRNA, RNA immunoprecipitation was performed using anti-MCPIP1 antibody. TaqMan analysis of the immunoprecipitated mRNAs showed that anti-MCPIP1 antibody pulled down larger amount of IL-6 mRNA than control IgG antibody did. To investigate whether there is any indirect effect on IL-6 mRNA stability upon knockdown of MCPIP1 through miRNAs, we investigated expression of several reported IL-6 targeting miRNAs (mir-26a, mir-26b, mir-142-3p and mir-146a). No significant difference in the expression of assayed miRNAs was observed. Finally, we determined the expression of MCPIP1 in damaged and smooth cartilage of OA (n = 9). In majority of samples (n = 7) MCPIP1 expression was down-regulated in damaged cartilage compared to smooth cartilage, suggesting lower expression of MCPIP1 may be contributing to the excessive expression of IL-6 in OA.
Conclusions: Expression of MCPIP1 in human cartilage and chondrocytes is shown for the first time. Differential expression of MCPIP1 in damaged and smooth cartilage and its stimulation by IL-1β, a pro-inflammatory cytokine implicated in the pathogenesis of the disease, suggest a role for MCPIP1 in OA. Interestingly binding of MCPIP1 protein with IL-6 mRNA indicated that enzymatic activity of MCPIP1 is important for the regulation of IL-6 mRNA expression in human chondrocytes. Moreover, expression of several reported IL-6 targeting miRNAs was unchanged upon MCPIP1 knockdown suggesting that MCPIP1 exert its effect directly on IL-6 mRNA. Furthermore, we also showed down-regulation of MCPIP1 expression in damaged cartilage compare to smooth cartilage suggesting its contribution in the up-regulation of IL-6 and related OA pathophysiology. Taken together, our data suggests that MCPIP1, a novel RNA modifying enzyme, may be an important player in OA pathogenesis.

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DNA OXIDATIVE DAMAGE AND ITS REPAIR ENZYME (OGG1) IN OSTEOARTHRITIC CHONDROCYTES

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Background: It is well known that chondrocytes produce excess amounts of reactive oxygen species (ROS) as well as proinflammatory cytokines and chemokines in response to mechanical and chemical stresses. Studies have provided ample confirmation of the generation of ROS and the depletion of cellular antioxidants in degenerated articular cartilage. 8-Oxoguanine, an oxidized form of guanine, is produced by ROS in large amounts in both DNA and nucleotide pools. 8-Oxoguanine is a major causative lesion for mutagenesis by ROS, since it can form a stable base pair with adenine as well as with cytosine during DNA replication. 8-Oxoguanine DNA glycosylase (Ogg1) repairs 8-oxoguanine, one of the most abundant DNA adducts caused by oxygen free radicals. Ogg1 is thought to protect against activation of the intrinsic apoptotic pathway in response to oxidative stress by augmenting DNA repair in a variety of cells. We postulated that depletion of cellular antioxidant, Ogg1, in osteoarthritic chondrocytes may participate in the degeneration of articular cartilage in OA. In the previous studies, we have focused on nanocarbon molecule, fullerene (C60), which acts a strong free radical scavenger, as an anti-oxidative agent, to protect against the OA relating catabolic stress-induced degeneration of articular cartilage both *in vitro* and *in vivo* OA models. We postulated that C60 may have a potential to protect against the catabolic process of articular cartilage through the mechanism involving the activation of Ogg1 in osteoarthritic chondrocytes.
Purpose: 1) The aim of the study was to examine the potential involvement of accumulation of 8-oxoguanine and impairment of DNA repair enzyme Ogg1 in the pathogenesis of OA.
2) Secondly, we studied whether or not C60 induced the expression and activation of Ogg1 in chondrocytes and protect against the down-regulation of OA chondrocytes and degeneration of articular cartilage *in vitro and in vivo*.
Methods: 8-Oxoguanine and Ogg1 expressions were immunohistorogically investigated in articular cartilages from patients with OA and from OA model rabbits. We studied whether OA-relating catabolic factors, IL-1beta (10 ng/ml) and H_2O_2 (100 μM), induced Ogg1 expression in OA chondrocytes. Knockdown of Ogg1 was also performed to investigate whether Ogg1 and C60 were associated with cellular activity and survival in human chondrocytes *in vitro*.
Results: The increased level of 8-oxoguanine and decreased level of Ogg1 in osteoarthritic chondrocytes of degenerated cartilage were observed in comparison with chondrocytes of intact cartilage in animal models of OA and in patients with OA. We also found that overexpression of Ogg1 in human osteoarthritis chondrocytes prevented