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Journal of Cellular Biochemistry 112:2813–2824 (2011)
Journal of Cellular Biochemistry

Role of Glucose as a Modulator of Anabolic and Catabolic Gene Expression in Normal and Osteoarthritic Human Chondrocytes

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ABSTRACT

Cartilage matrix homeostasis involves a dynamic balance between numerous signals that modulate chondrocyte functions. This study aimed at elucidating the role of the extracellular glucose concentration in modulating anabolic and catabolic gene expression in normal and osteoarthritic (OA) human chondrocytes and its ability to modify the gene expression responses induced by pro-anabolic stimuli, namely Transforming Growth Factor-β (TGF). For this, we analyzed by real time RT-PCR the expression of articular cartilage matrix-specific and non-specific genes, namely collagen types II and I, respectively. The expression of the matrix metalloproteinases (MMPs)-1 and -13, which plays a major role in cartilage degradation in arthritic conditions, and of their tissue inhibitors (TIMP) was also measured. The results showed that exposure to high glucose (30 mM) increased the mRNA levels of both MMPs in OA chondrocytes, whereas in normal ones only MMP-1 increased. Collagen II mRNA was similarly increased in normal and OA chondrocytes, but the increase lasted longer in the later. Exposure to high glucose for 24 h prevented TGF-induced downregulation of MMP-13 gene expression in normal and OA chondrocytes, while the inhibitory effect of TGF on MMP-1 expression was only partially reduced. Other responses were not significantly modified. In conclusion, exposure of human chondrocytes to high glucose, as occurs in vivo in diabetes mellitus patients and in vitro for the production of engineered cartilage, favors the chondrocyte catabolic program. This may promote articular cartilage degradation, facilitating OA development and/or progression, as well as compromise the quality and consequent in vivo efficacy of tissue engineered cartilage. J. Cell. Biochem. 112: 2813–2824, 2011.

KEY WORDS: COLLAGEN; GENE EXPRESSION; GLUCOSE; HUMAN CHONDROCYTE; MMP; OSTEOARTHRITIS; TIMP

Articular cartilage is a specialized connective tissue that supports and distributes loads and ensures a near-frictionless motion in joints. These unique properties are due to the structural organization of the main macromolecules that compose the cartilage extracellular matrix, namely collagens and proteoglycans. Chondrocytes, the only cell type present in articular cartilage, are embedded in the extracellular matrix and are responsible for maintaining its homeostasis by ensuring the synthesis and turnover of its components [Martel-Pelletier et al., 2008; Goldring and Marcu, 2009].

Cartilage matrix homeostasis involves a dynamic balance between a variety of signals that modulate chondrocyte functions, namely mechanical forces, cytokines and growth factors and cell–matrix interactions, some favoring an anabolic program and others

Grant sponsor: Portuguese Foundation for Science and Technology (FCT); Grant number: PTDC/SAU-OSM/67936/2006; Grant sponsor: FCT; Grant numbers: SFRH/BD/19763/2004, SFRH/BD/47470/2008.

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Received 16 September 2010; Accepted 13 May 2011 • DOI 10.1002/jcb.23196 • © 2011 Wiley-Liss, Inc.
Published online 23 May 2011 in Wiley Online Library (wileyonlinelibrary.com).

stimulating catabolic responses. Aging and mechanical stress of joints are major risk factors for osteoarthritis (OA), but growing evidence indicates that metabolic factors play an important role in disease development and progression. For instance, a significant positive correlation was found between elevated blood glucose and radiological evidence of OA [Hart et al., 1995]. Accordingly, OA is increasingly envisaged as a ''metabolic disorder'' linked to obesity, diabetes mellitus, dyslipidemia, hypertension, and insulin resistance [Sturmer et al., 2001; Burner and Rosenthal, 2009; Siviero et al., 2009; Pallu et al., 2010; Velasquez and Katz, 2010].

These conditions seem to favor the initiation and progression of arthritic diseases, such as OA, by providing catabolic signals that lead, among other responses, to increased production of proteolytic enzymes. These degrade the matrix components causing progressive cartilage destruction, the main feature of OA [Martel-Pelletier et al., 2008; Goldring and Marcu, 2009].

Among the proteolytic enzymes, aggrecanases and colagenases which belong to the Matrix MetalloProteases (MMPs) family are of special importance [Arner, 2002; Nagase and Kashiwagi, 2003; Burrage et al., 2006]. Collagenases cleave collagen triple helix fibers, leaving them susceptible to further degradation by other enzymes. Human articular chondrocytes have been shown to express several collagenases, among which collagenase-1 (MMP-1), -2 (MMP-8), and -3 (MMP-13) are especially relevant since their expression is increased by pro-inflammatory and catabolic stimuli [Arner, 2002; Nagase and Kashiwagi, 2003; Burrage et al., 2006]. MMP-13, whose expression has been shown to be augmented in OA cartilage [Bau et al., 2002; Kevorkian et al., 2004], has a significant role in cartilage degradation and OA progression since it preferentially cleaves type II collagen fibers [Takaishi et al., 2008]. MMP-1, also participates in the initial cleavage of collagen II, but with a lower activity than MMP-13 [Mitchell et al., 1996]. Type II collagen is a major macromolecule of the extracellular matrix that confers structural support to cartilage. Its enhanced degradation and consequent loss with associated cartilage erosion is a key characteristic of OA pathophysiology. Allied to increased matrix degradation, OA is characterized by a shift in the chondrocyte anabolic activity, so that collagen II synthesis is replaced by increased production of the non-cartilage matrix specific collagen type I. This leads to the formation of a fibrocartilaginous tissue which is biomechanically less effective than articular cartilage [Miosge et al., 2004; Tesche and Miosge, 2005].

The activity of MMPs can be negatively regulated at the post-transcriptional level by members of the family of Tissue Inhibitors of MetalloProteases (TIMPs) [Baker et al., 2002]. Among the TIMPs identified in human chondrocytes, TIMP-2 has been reported to be constitutively expressed and therefore suggested to have a role in the maintenance of cartilage integrity in normal conditions, while TIMP-1 and TIMP-3 seem to have a more important role in pathological conditions [Zafarullah et al., 1996; Su et al., 1999]. High ratios of proteinase/inhibitors favor proteolysis and have been found in the synovial fluid of OA patients [Ishiguro et al., 1999].

Members of the MMP and TIMP families are inversely regulated at the transcriptional level by catabolic stimuli, such as the pro-inflammatory cytokines, Interleukin-1β (IL-1) and Tumor Necrosis Factor-α (TNF), and by anabolic growth factors, namely Transforming Growth Factor-β (TGF). The last is especially important since it can act in articular chondrocytes in an autocrine and/or paracrine way, inducing anabolic effects and inhibiting catabolic responses. More specifically, TGF has been shown to stimulate proteoglycans, collagen type II [Miyazaki et al., 2000; Martel-Pelletier et al., 2008] and TIMPs expression [Wang et al., 2002], while reversing some of the IL-1-induced catabolic effects [Seifarth et al., 2009].

Besides these well-characterized stimuli, chondrocytes respond to and are influenced by other signals. Among these, the extracellular glucose concentration, either increased or decreased, has been shown to directly affect some chondrocyte functions [Kelley et al., 1999; Richardson et al., 2003; McNulty et al., 2005]. In particular, culture of rabbit chondrocytes in either low (<5 mM) or high (25 mM) glucose concentrations decreased proteoglycan synthesis induced by the anabolic growth factor, IGF-I [Kelley et al., 1999]. Exposure to increasing glucose concentrations, dose-dependently decreased dehydroascorbate uptake by human chondrocytes [McNulty et al., 2005], which may have functional implications as dehydroascorbate is essential for collagen synthesis. Moreover, our previous study showed that normal human chondrocytes adjust to changes in the extracellular glucose concentration by regulating glucose uptake, at least in part, through modulation of the cellular content of the Glucose Transporter (GLUT)-1 [Rosa et al., 2009], a member of the GLUT/SLC2A facilitative glucose transporter family. Moreover, that study also showed that OA chondrocytes exposed to a high, hyperglycemia-like extracellular glucose concentration (30 mM) were unable to reduce glucose transport and GLUT-1 content which led to increased glucose accumulation and prolonged Reactive Oxygen Species (ROS) production [Rosa et al., 2009]. Increased oxidative stress has been pointed out as a major pathogenic mechanism of OA [Maneiro et al., 2003; Grishko et al., 2009], and at the cellular level, increased ROS production has been shown to mediate many catabolic responses in chondrocytes [Lo et al., 1998; Mendes et al., 2003a,b].

Thus, this study was undertaken to determine whether exposure to a high extracellular glucose concentration can shift normal and OA chondrocyte functions towards a pro-catabolic and/or anti-anabolic gene expression profile, either direct or indirectly by modifying the responses induced by pro-anabolic stimuli, namely TGF.

For this purpose, we examined by real time RT-PCR (qRT-PCR) the expression of genes that are important for cartilage homeostasis and OA pathogenesis and that in other cell types have been reported to be modulated by exposure to high glucose [Death et al., 2003; Ho et al., 2007; Andreea et al., 2008; Kim et al., 2008]. Those genes include the MMPs-1 and -13 due to their major role in cartilage matrix degradation, and collagen type II, TIMP-1 and TIMP-2 as important anabolic and anti-catabolic genes, respectively. Collagen type I was also assessed since it is frequently reported to be increased in OA, representing the inability of OA chondrocytes to promote an adequate cartilage matrix repair response [Miosge et al., 2004].

MATERIALS AND METHODS

CARTILAGE SAMPLES AND CHONDROCYTE CULTURE

Human knee cartilage was collected, within 12 h of death, from the distal femoral condyles of multi-organ donors (18–40 years old,

mean = 32, n = 7, normal cartilage) or with informed consent from patients (50–71 years old, mean = 64, n = 11, OA cartilage) undergoing total knee replacement surgery at the Orthopedic Department of the University Hospital of Coimbra (HUC). The Ethics Committee of HUC approved all procedures. Cartilage from all multi-organ donors appeared grossly normal without macroscopic changes other than some yellowish discoloration and, in a few samples (aged >35 years old), small areas of slightly roughened surface. Chondrocytes were isolated by enzymatic digestion as described previously [Rosa et al., 2009]. Non-proliferating monolayer cultures were established from each cartilage sample, allowed to recover in medium containing 5% fetal bovine serum for 24 h, serum-starved overnight and maintained thereafter in serum-free culture medium. The cells were subsequently cultured, for the periods indicated in figures and figure legends, in Ham's F-12 (Regular Glucose Medium, which contains 10 mM glucose) or in the same medium supplemented with 20 mM D-glucose to yield a final glucose concentration of 30 mM (High Glucose Medium). In selected experiments, recombinant human TGF-β (Peprotech, Rocky Hill, NJ) was used in the final concentration of 10 ng/ml.

TOTAL RNA EXTRACTION AND cDNA PREPARATION

Total RNA was extracted with TRIzol (Invitrogen, Paisley, UK), analyzed using Experion RNA StdSens Chip (Bio-Rad) and quantified in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) at 260 nm. The cDNA was reverse transcribed from 1 μg of total RNA, using iScriptTM Select cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions. The cDNA obtained was stored at −20°C until further analysis.

REAL-TIME REVERSE TRANSCRIPTASE-PCR (qRT-PCR)

Specific sets of primers were designed using Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA). Details of the forward and reverse primers for the human genes evaluated are presented in Table I. qRT-PCR was performed with iTaq^{TM} DNA polymerase using iQ^{TM} SYBR Green Supermix (BioRad). Thermal cycling conditions included 3 min at 95°C to activate the iTaq^{TM} DNA polymerase, followed by 45 cycles, each consisting of a denaturation step at 95°C for 10 s, an annealing step at 54°C for 30 s and an elongation step at 72°C for 30 s. Fluorescence measurements were taken every cycle at the end of the annealing step and the specificity of the amplification products was checked by analysis of the melting curve. The efficiency of the amplification reaction for each gene was calculated by running a standard curve of serially diluted cDNA sample. In each assay, a control reaction without the cDNA was also subjected to PCR amplification.

Gene expression changes were analyzed using the built-in iQ5 Optical system software v2, which enables the analysis of the results with the Pfaffl method, a variation of the ΔΔCT method corrected for gene-specific efficiencies.

STATISTICAL ANALYSIS

In Figure 1, the nonparametric Mann–Whitney test was used to compare the distributions of the basal expression of catabolic and anabolic genes in normal and OA human chondrocytes. In all other figures, the results are presented as the gene expression level relative to the control situation (normal or OA chondrocytes maintained in RGM). The statistical analysis was performed using the paired two-tailed Student *t*-test to compare differences between the control and the test situation or between the responses induced by treatment of chondrocytes with TGF in RGM and in HGM, for the same incubation period. To assess normality for the observed differences, a graphical analysis based on normal quantile plots was used in all cases, showing no strong departure from normality, thus supporting the use of the Student *t*-test. Results were considered statistically significant for $P < 0.05$.

RESULTS

BASAL EXPRESSION OF ANABOLIC AND CATABOLIC GENES IN NORMAL AND OA HUMAN CHONDROCYTES

The expression of MMPs-1 and -13, TIMPs-1 and -2 and collagen types I and II was analyzed by qRT-PCR across cDNAs transcribed from total RNA extracted from chondrocyte cultures obtained from 7 normal and 11 OA human knee cartilage samples. Previous studies showed that HPRT-1 was the candidate housekeeping gene, among seven tested, whose expression varied least between normal and OA chondrocytes. Accordingly, HPRT-1 was used as reference gene for comparisons of mRNA levels between normal and OA chondrocytes.

TABLE I. Oligonucleotide Primer Pairs Used for qRT-PCR

Gene name	Primer sequences (5′–3′)	RefSeq ID
Hypoxanthine phosphoribosyltransferase-1 (HPRT-1)	F: TGA CAC TGG CAA AAC AAT R: GGC TTA TAT CCA ACA CTT CG	NM_000194
Matrix Metalloprotease-1 (MMP-1)	F: GACTCTCCCATTCTACTG R: TTA TAG CAT CAA AGG TTA GC	NM_002421
Matrix Metalloprotease-13 (MMP-13)	F: GTT TCC TAT CTA CAC CTA CAC R: CTC GGA GAC TGG TAA TGG	NM_002427
Tissue inhibitor of metalloproteinase-1 (TIMP-1)	F: TGTTGCTGTGGCTGATAG R: CTGGTATAAGGTGGTCTGG	NM_003254
Tissue inhibitor of metalloproteinase-2 (TIMP-2)	F: ACGATATACAGGCACATTATG R: GGTCAGGAGTCTTAACAGG	NM_003255
Collagen Type I (COL1A1)	F: GGA GGA GAG TCA GGA AGG R: GCA ACA CAG TTA CAC AAG G	NM_000088
Collagen type II (COL2A1)	F: GGC AGA GGT ATA ATG ATA AGG R: ATT ATG TCG TCG CAG AGG	NM_001844

F: Forward sequence; R: Reverse sequence.

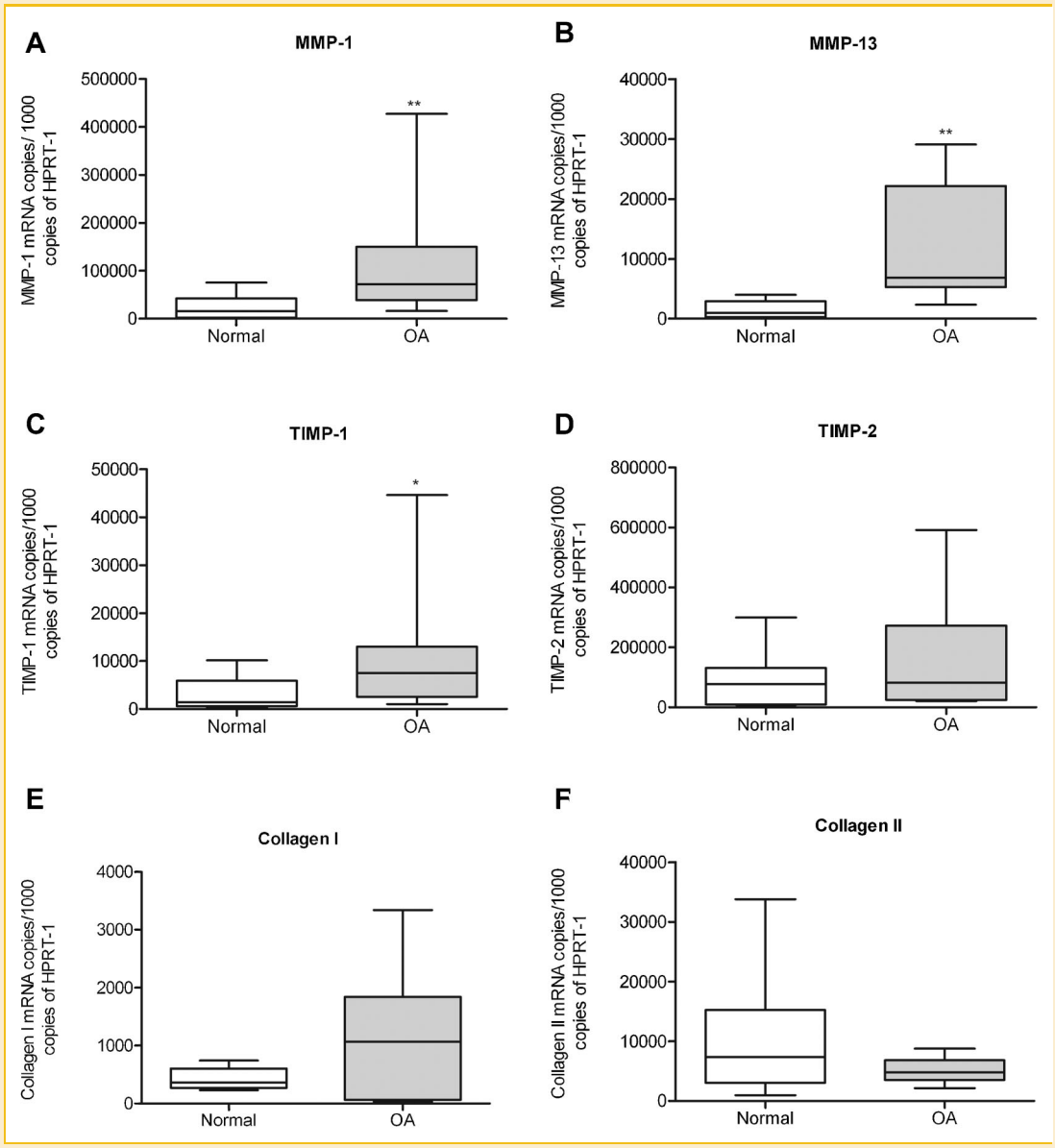

Fig. 1. Basal expression of catabolic and anabolic genes in normal and OA human chondrocytes. Box and whiskers plots of the mRNA levels of MMPs-1 (A) and -13 (B), TIMPs-1 (C) and -2 (D) and collagens I (E) and II (F) in normal ($n = 7$) and OA ($n = 11$) chondrocytes. The boxes represent the distribution of mRNA levels between the 25th and 75th percentiles, while the bars (whiskers) represent extreme values outside that range. The line dividing each box represents the median of the distribution, that is, the central mRNA value for which there is an equal number of samples with mRNA values above and below that one. $^{*}P < 0.05$ and $^{**}P < 0.01$.

The box and whiskers plots presented in Figure 1A and B show that, despite considerable interindividual variability and a few outliers in both groups, basal MMP-1 and -13 mRNA levels were significantly higher in OA than in normal chondrocytes. On average, MMP-1 and -13 mRNA levels were approximately five and eight times higher in OA ($118{,}011.7 \pm 39{,}681.4$ and $11{,}926.9 \pm 3{,}215.4$) than in normal ($24{,}243.6 \pm 10{,}100.4$ and $1{,}469.7 \pm 694.9$) chondrocytes, respectively. In all samples tested, either normal or OA, MMP-1 was always detected at an earlier mean threshold cycle than MMP-13, indicating a higher basal expression level of the former.

On the other hand, the expression of TIMP-1 was significantly higher (approximately fourfold in average) in OA than in normal chondrocytes (Fig. 1C, $P = 0.04$). TIMP-2 mRNA levels, however, were only 1.7-fold higher in OA than in normal chondrocytes and the difference did not reach statistical significance (Fig. 1D, $P = 0.54$).

Concerning the mRNA expression of collagens I and II, a tendency to increased levels of collagen II in normal and to elevated levels of collagen I in OA chondrocytes was observed (Fig. 1E and F), although those differences did not reach statistical significance. Moreover, the ratio of collagen II over collagen I mRNA levels (Col

II/Col I) was on average 3.5-fold higher in normal than in OA chondrocytes, but again the difference did not reach statistical significance ($P = 0.07$).

ROLE OF HIGH GLUCOSE IN MODULATING THE EXPRESSION OF THE CATABOLIC GENES, MMP-1 AND -13

The results obtained show that in normal chondrocytes, MMP-1 mRNA levels (Fig. 2A) relative to chondrocytes cultured in regular glucose medium (RGM, 10 mM glucose) decreased upon exposure to high glucose (HGM, 30 mM glucose) for 48 and 72 h, while MMP-13 mRNA levels (Fig. 2C) remained unchanged regardless of the duration of exposure to high glucose. In OA chondrocytes, both MMP-1 (Fig. 2B) and -13 (Fig. 2D) mRNA levels were similarly increased by culture in HGM for 24, 48, and 72 h. Nonetheless, the differences observed for MMP-1 at 24 and 48 h did not reach statistical significance.

ROLE OF HIGH GLUCOSE ON TIMP-1 AND -2 EXPRESSION

The results shown in Figure 3A indicate that the expression of TIMP-1 was decreased by culture of normal chondrocytes in HGM for 48 and 72 h, while no significant changes were found in OA chondrocytes (Fig. 3B). TIMP-2 mRNA levels were significantly upregulated in normal (Fig. 3C), but not in OA (Fig. 3D) chondrocytes upon exposure to high glucose for 24 h. No significant

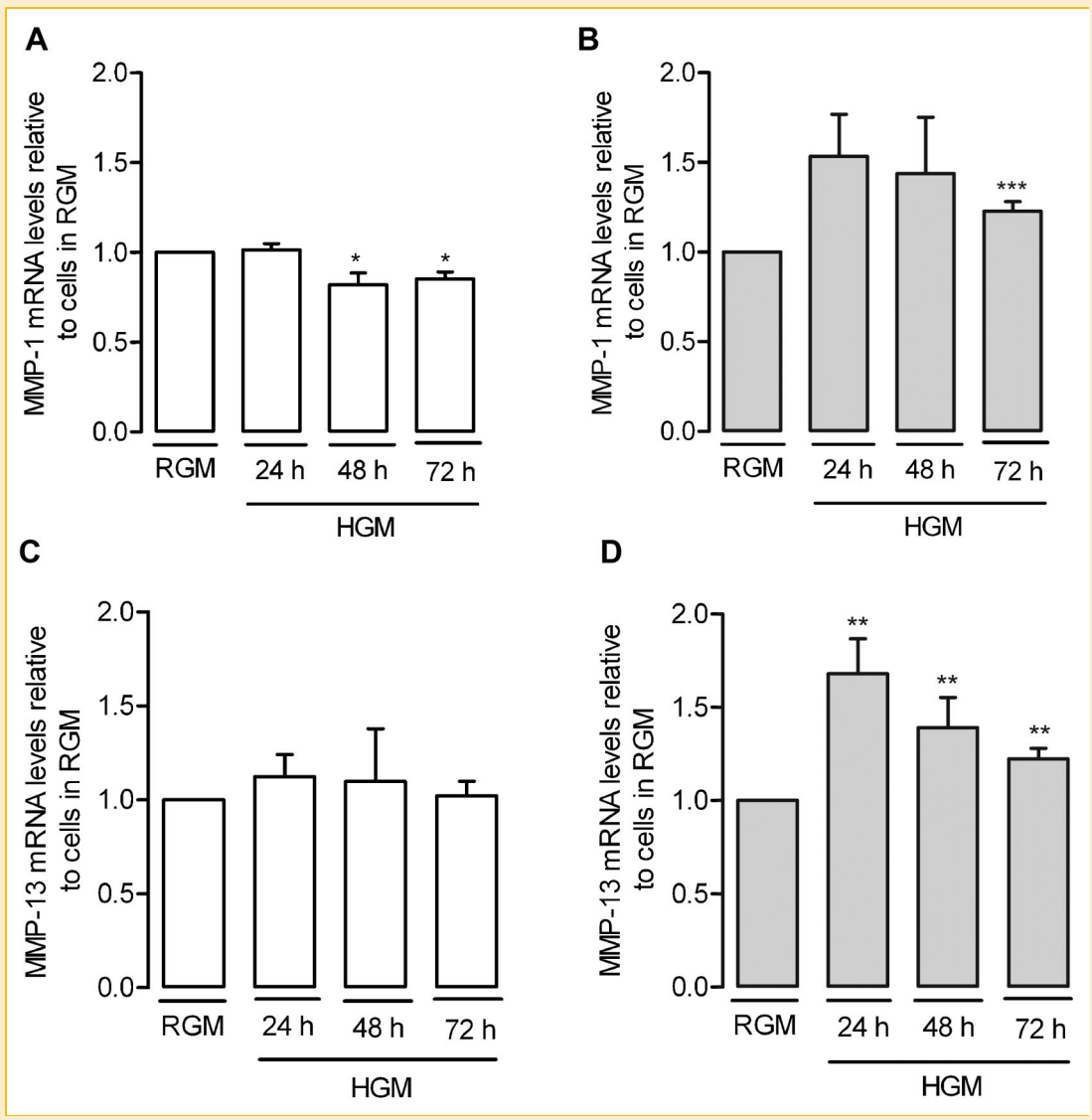

Fig. 2. Role of high glucose in modulating the expression of MMP-1 and MMP-13 genes in normal and OA chondrocytes. Relative MMP-1 (A, B) and MMP-13 (C, D) mRNA levels in normal ($n = 5$) and OA ($n = 6$) chondrocytes cultured in medium with an elevated (30 mM) glucose concentration (HGM) for 24, 48, and 72 h or maintained in medium with the regular glucose concentration (RGM) for the entire experiment (normal and OA controls). Each bar represents the mean ± SD. $^{*}P < 0.05$ and $^{***}P < 0.001$ relative to the respective control.

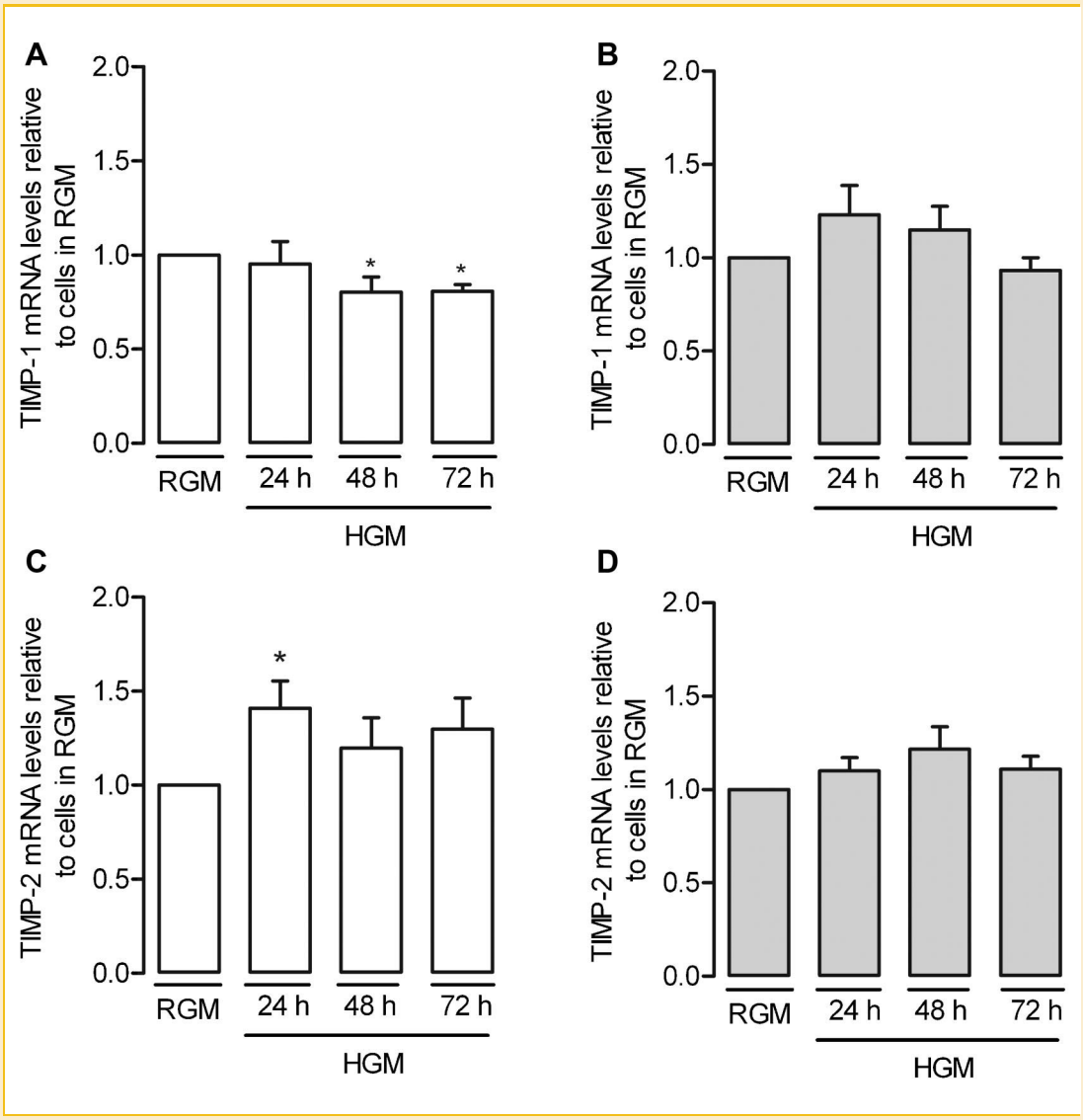

Fig. 3. Role of high glucose in modulating the expression of TIMP-1 and TIMP-2 genes in normal and OA chondrocytes. Relative TIMP-1 (A, B) and TIMP-2 (C, D) mRNA levels in normal ($n = 6$) and OA ($n = 6$) chondrocytes cultured in medium with an elevated (30 mM) glucose concentration (HGM) for 24, 48, and 72 h or maintained in medium with the regular glucose concentration (RGM) for the entire experiment (normal and OA controls). Each bar represents the mean ± SD. $^{*}P < 0.05$ relative to the respective control.

differences relative to cells maintained in RGM were found at the other time points, either in normal (Fig. 3C) or OA chondrocytes (Fig. 3D).

ROLE OF HIGH GLUCOSE ON COLLAGEN TYPE I AND II EXPRESSION

Figure 4A and B shows that culture of normal and OA chondrocytes, respectively, in HGM for 24, 48, or 72 h did not significantly affect collagen type I expression relative to the respective control cells maintained in RGM.

On the other hand, culture in HGM for 24 h similarly increased collagen II mRNA levels in normal (1.3 ± 0.07, Fig. 4C) and OA (1.3 ± 0.13, Fig. 4D) chondrocyte cultures. This increase, although lower, was still significant after incubation of OA chondrocytes in HGM for 48 h. After 72 h, however, collagen II mRNA levels both in normal and OA chondrocytes had returned to those found in the respective control cells maintained in RGM.

ROLE OF HIGH GLUCOSE IN MODULATING MMP-1 AND -13 GENE EXPRESSION PROFILES INDUCED BY TGF IN NORMAL AND OA CHONDROCYTES

To determine whether exposure to high glucose can modify chondrocyte responses to TGF, the gene expression profiles of MMP-1 and -13 were evaluated in normal and OA chondrocytes treated with TGF for various time periods in the presence or absence of high glucose (HGM). The results were normalized using HPRT-1 as the housekeeping gene since its expression was not significantly affected by treatment of normal and OA chondrocytes with TGF.

Treatment with TGF for 24 h induced a marked decrease in MMP-1 mRNA levels in normal (0.31 ± 0.015, Fig. 5A) and OA (0.38 ± 0.05,

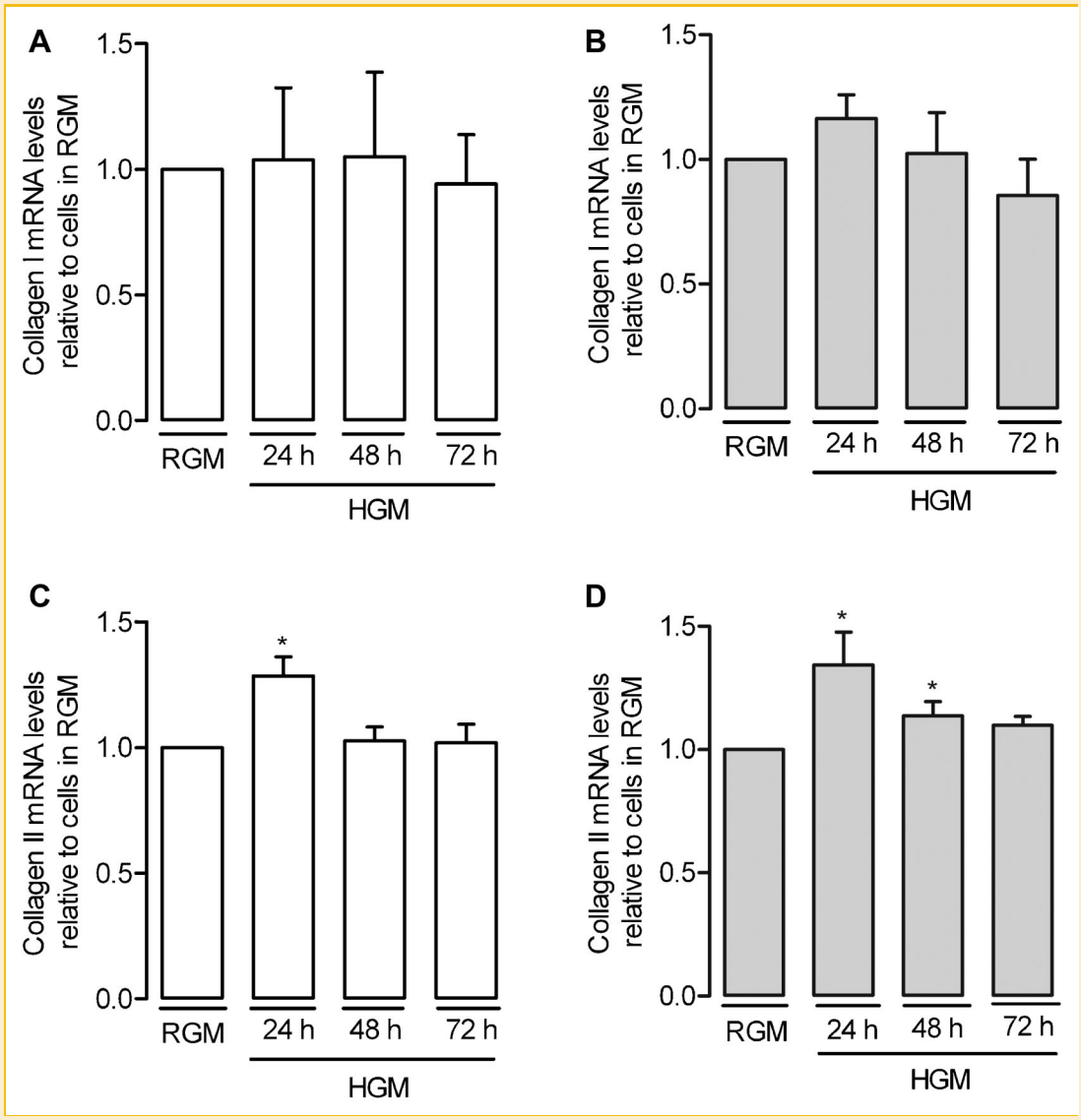

Fig. 4. Role of high glucose in modulating the expression of collagen type I and II genes in normal and OA chondrocytes. Relative collagen type I (A, B) and collagen type II (C, D) mRNA levels in normal ($n = 5$) and OA ($n = 5$) chondrocytes cultured in medium with an elevated (30 mM) glucose concentration (HGM) for 24, 48, and 72 h or maintained in medium with the regular glucose concentration (RGM) for the entire experiment (normal and OA controls). Each bar represents the mean ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ relative to the respective control.

Fig. 5B) chondrocytes relative to the respective controls maintained in RGM, which persisted up to, at least, 72 h. Yet, exposure to high glucose for 24 and 72 h significantly reduced the inhibitory effect of TGF on MMP-1 expression, either in normal or OA chondrocytes (Fig. 5A and B, respectively), which corresponds to higher MMP-1 mRNA levels. At 48 h, no significant differences were found between the responses to TGF treatment in the presence or absence of HGM, either in normal or in OA chondrocytes.

Moreover, treatment of normal (Fig. 5C) and OA (Fig. 5D) human chondrocytes with TGF in RGM induced a marked and sustained decrease in MMP-13 expression that was maximal at 72 h (0.33 ± 0.11 and 0.25 ± 0.09, respectively). Under high glucose (HGM), however, the inhibitory effect of TGF treatment was abolished, but the kinetics in normal and OA chondrocytes were not identical. Indeed, in normal chondrocytes, exposure to high glucose for 24 h completely reversed the effect of TGF on MMP-13 mRNA levels, but more prolonged culture periods under high glucose, namely 48 and 72 h, no longer significantly affected the response to TGF. In OA chondrocytes cultured in HGM for 24 h, the response to TGF was completely abolished and still significant at 48 h. Full recovery of the ability of TGF to reduce MMP-13 mRNA levels in the presence of HGM occurred only upon culture for 72 h.

ROLE OF HIGH GLUCOSE IN MODULATING TGF-INDUCED TIMP-1 AND -2 GENE EXPRESSION IN NORMAL AND OA CHONDROCYTES

Figure 6A shows that in normal chondrocytes, TIMP-1 expression was only slight and transiently increased by treatment with TGF for 48 h in RGM and was not modified by exposure to high glucose. In OA

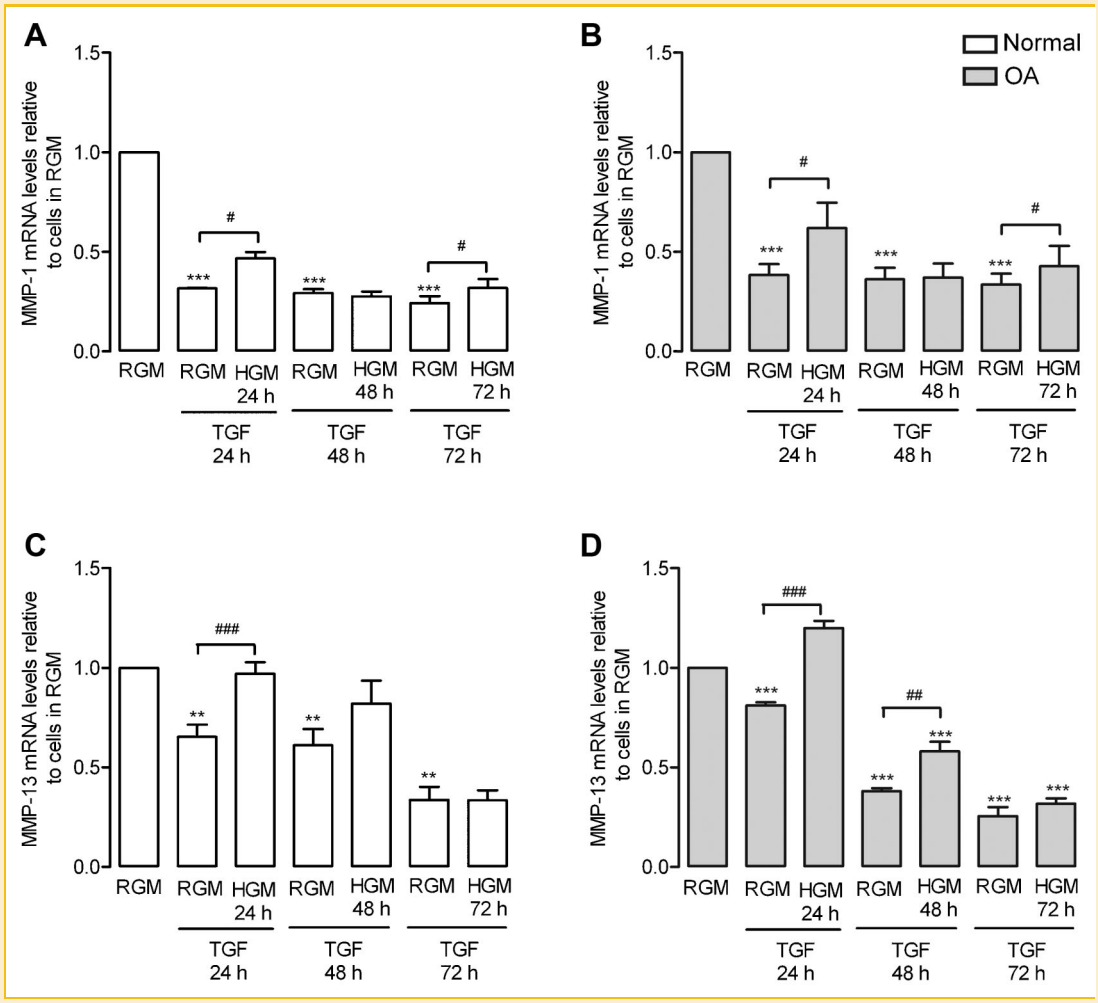

Fig. 5. Role of high glucose in modulating MMP-1 and -13 gene expression induced by TGF in normal and OA chondrocytes. Relative mRNA levels of MMP-1 (A, B) and MMP-13 (C, D) in normal ($n = 5$) and OA ($n = 6$) chondrocytes. Chondrocytes were treated with TGF 10 ng/ml, for 24, 48, and 72 h in medium with a high (30 mM, HGM) or regular (10 mM, RGM) glucose concentration or left untreated under RGM (normal and OA controls). $^{**}P < 0.01$ and $^{***}P < 0.001$ relative to the respective control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ between TGF in RGM and in HGM for each time point.

chondrocytes, however, TIMP-1 expression (Fig. 6B) was significantly induced in a time-dependent manner upon TGF treatment, but independently of the glucose concentration in the culture medium.

In contrast, TGF induced TIMP-2 mRNA expression in normal chondrocytes (Fig. 6C) relative to the respective control cells. The observed increase was time-dependent and maximal at 72 h (2.7 ± 0.26). As shown in Figure 6D, in OA chondrocytes, TGF-induced TIMP-2 mRNA levels were similar and significantly augmented at the three time points, but the relative increase was lower than that observed in normal chondrocytes. Induction of TIMP-2 mRNA levels by TGF was not affected by the glucose concentration in the culture medium, either in normal or OA chondrocytes.

ROLE OF HIGH GLUCOSE IN MODULATING COLLAGEN I AND II GENE EXPRESSION INDUCED BY TGF IN NORMAL AND OA CHONDROCYTES

The results presented in Figure 7A and B show that treatment with TGF increased collagen I mRNA levels both in normal and OA chondrocytes and independently of the glucose concentration in the culture media. Interestingly, the relative increase in collagen I expression was significantly higher in OA than in normal chondrocytes. Indeed, treatment of this group with TGF for 24 and 48 h increased collagen I mRNA levels to 2.0 ± 0.2 and 2.1 ± 0.5 (Fig. 7A), respectively, whereas in OA chondrocytes the increases were 3.8 ± 0.8 and 2.5 ± 0.5 (Fig. 7B), respectively, relative to the corresponding cells maintained in RGM in the absence of TGF treatment. Additionally, collagen I mRNA levels remained significantly elevated in OA chondrocytes treated with TGF for 72 h (2.7 ± 0.8), while in normal chondrocytes those levels (1.6 ± 0.5) were not significantly different from the respective control group.

Treatment of normal chondrocytes with TGF for 24 and 48 h, regardless of the presence or absence of high glucose, increased collagen II mRNA levels, but the differences did not reach statistical significance (Fig. 7C). In contrast, collagen II expression in OA chondrocytes was significantly increased by TGF treatment (Fig. 7D). The relative increase in collagen II expression was

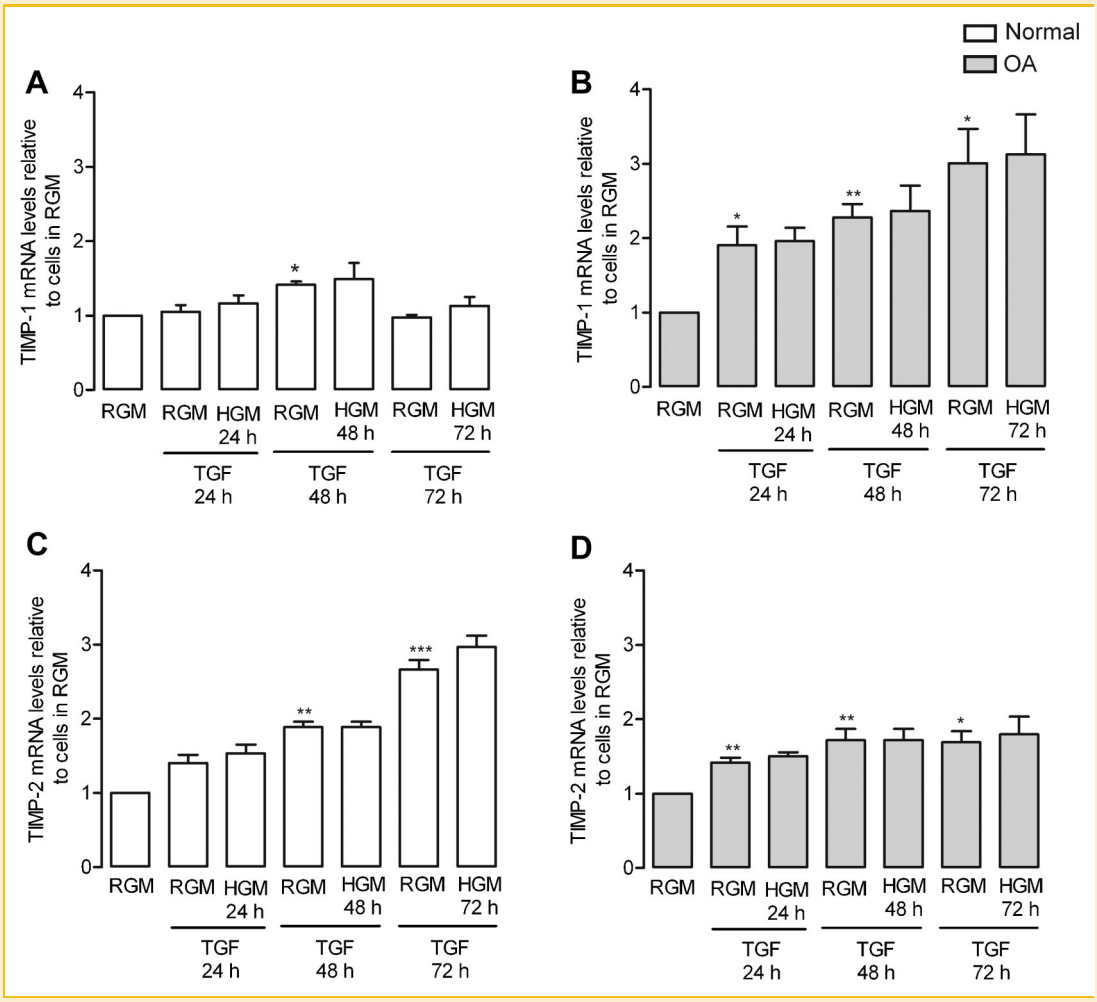

Fig. 6. Role of high glucose in modulating TIMP-1 and TIMP-2 gene expression induced by TGF in normal and OA chondrocytes. Relative mRNA levels of TIMP-1 (A, B) and TIMP-2 (C, D) in normal ($n = 6$) and OA ($n = 6$) chondrocytes. Chondrocytes were treated with TGF 10ng/ml for 24, 48, and 72 h in medium with a high (30 mM, HGM) or regular (10 mM, RGM) glucose concentration or left untreated under RGM (normal and OA controls). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ relative to the respective control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ between TGF in RGM and in HGM for each time point.

maximal at 24 h (2.2 ± 0.6), slightly decreasing thereafter (1.7 ± 0.3 and 2.1 ± 0.6 at 48 and 72 h, respectively). Culture under high glucose did not significantly alter the response to TGF, except at 72 h when a small, but statistically significant increase was found (2.5 ± 0.6, $P = 0.008$).

DISCUSSION

The results presented in Figure 1 are in agreement with previous reports showing that MMP-1 and -13 expression is higher in OA than in normal chondrocytes [Bau et al., 2002; Kevorkian et al., 2004]. Moreover, the average ratio of collagen type II expression over collagen type I (Col II/Col I) was found to be higher in normal than in OA chondrocytes, likely due to the shift towards increased collagen I and decreased collagen II expression in OA chondrocytes (Fig. 1E and F). Nonetheless, this difference did not reach statistical significance, reflecting a great variability between individuals in each group that only much larger cohorts would minimize. The Col II/Col I ratio has been defined as a chondrocyte differentiation index and used to characterize normal and OA cartilage and the differentiation state of monolayer chondrocyte cultures, where lower ratios correspond to a less differentiated phenotype [Martin et al., 2001; Marlovits et al., 2004]. Even so, since we could not obtain age-matched normal and OA cartilage samples and given the relatively large age range in each group, the possibility that the differences observed are age-related cannot be excluded.

The major purpose of this study was to determine whether variations in the extracellular glucose concentration affect chondrocyte anabolic and catabolic gene expression. The results obtained show that human chondrocytes respond to the extracellular glucose concentration which can modulate anabolic and catabolic responses, both directly and by modifying the response to anabolic growth factors, namely TGF. In particular, this study shows that exposure of OA chondrocytes to high glucose (30 mM) increased MMP-1 and -13 mRNA expression and slight and

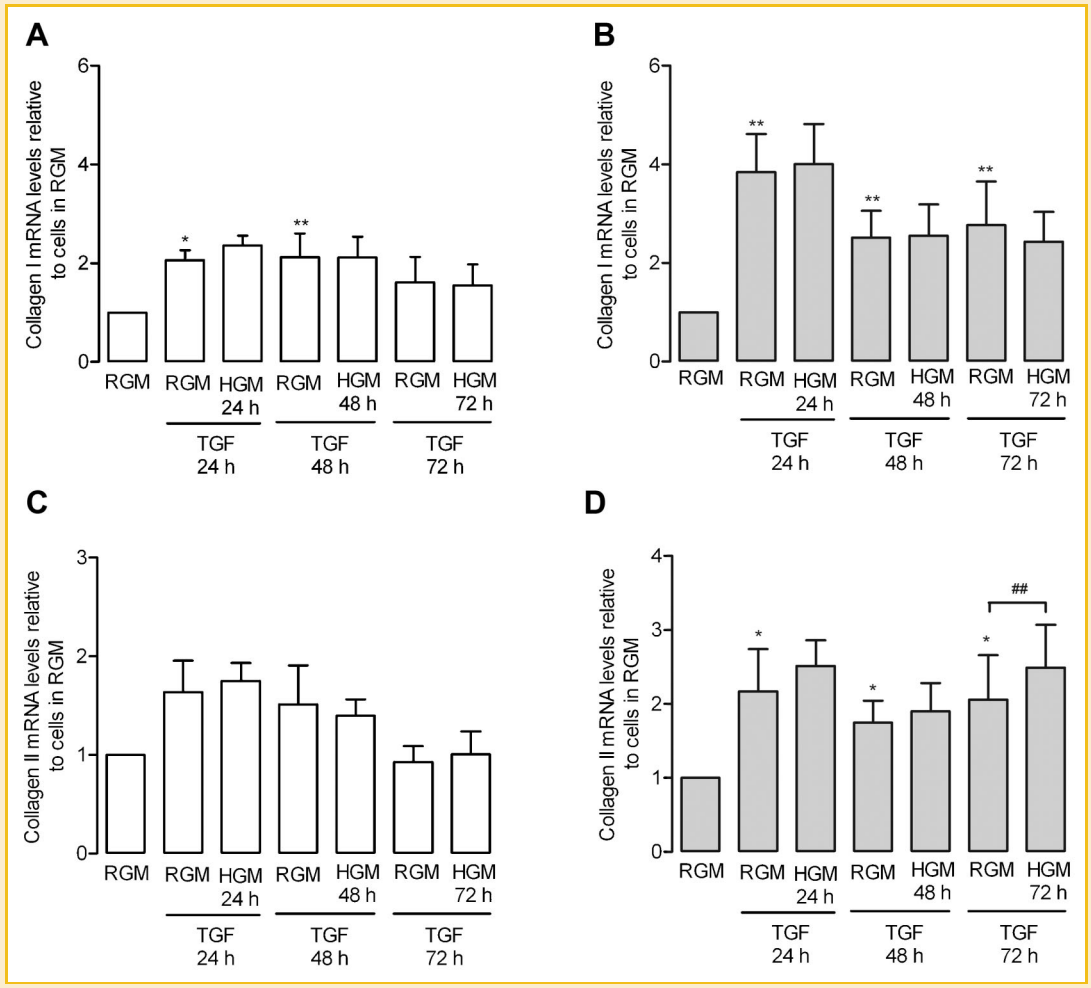

Fig. 7. Role of high glucose in modulating Collagens type I and II gene expression induced by TGF in normal and OA chondrocytes. Relative mRNA levels of collagen I (A, B) and collagen II (C, D) in normal (n = 5) and OA (n = 5) chondrocytes treated with TGF, 10 ng/ml, for 24, 48, and 72 h in medium with a high (30 mM, HGM) or regular (10 mM, RGM) glucose concentration or left untreated under RGM (normal and OA controls). $^{*}P < 0.05$ and $^{**}P < 0.01$ relative to the respective control; $^{\#}P < 0.01$ between TGF in RGM and in HGM for each time point.

transiently augmented collagen II expression, while TIMP-1, TIMP-2 and collagen I were not affected. In contrast, the expression of MMP-13 and collagen I was not affected by high glucose in normal chondrocytes, whereas MMP-1 and TIMP-1 expression were decreased and TIMP-2 and collagen II levels were transitorily increased. These results indicate that exposure to high glucose can promote both catabolic and anabolic responses in human chondrocytes, but OA chondrocytes are more susceptible to deleterious effects than their normal counterparts. This is in agreement with our previous study showing that OA chondrocytes fail to downregulate glucose transport and produce more ROS over longer periods when exposed to high glucose [Rosa et al., 2009].

On the other hand, most of the effects induced by exposure of either normal or OA chondrocytes to high glucose were transient. Two mechanisms may contribute to the reversibility of those effects. On one hand, glucose concentration in the culture medium decreases over time due to cell consumption, which may cause the initial effect to gradually wear out. On the other hand, it is also possible that the effects at the level of gene expression are transient, while at the protein level the alterations last longer. Although beyond its scope, this study paves the way for future research on the role of high glucose in modulating the protein levels of the genes whose expression was found to be altered, as well as of other proteins relevant in chondrocyte biology and arthritis pathology.

Moreover, the observation that in OA chondrocytes high glucose-induced MMP-1 and -13 mRNA expression was not accompanied by increased TIMP-1 and -2 expression, suggests that the uncoordinated expression of the MMP/TIMP system can lead to a greater imbalance in the activity of the former, favoring matrix degradation and further compromising OA cartilage integrity. Therefore, this may represent a relevant pathological mechanism by which high glucose concentrations can contribute to OA pathology. On the other hand, exposure to a high glucose concentration did not affect TGF-induced anabolic responses, except for a slight increase in collagen II mRNA levels (Fig. 7D), but it did reverse TGF-induced anti-catabolic gene expression. In particular, elevated glucose inhibited TGF-induced decrease in MMP-13 expression both in normal and OA chondrocytes, although the effect was more prolonged in OA

chondrocytes (Fig. 5C and D). This may constitute another mechanism by which high glucose concentrations can cause further damage to chondrocytes and articular cartilage. The oxidative stress induced by high glucose in OA and also in normal chondrocytes, although for significantly shorter periods in the later [Rosa et al., 2009], may impair normal TGF signaling and consequent responses. Indeed, increased ROS production has been shown to induce the gene expression of various MMPs in chondrocytes from different species [Lo et al., 1998; Gurjar et al., 2001]. Thus, it is possible that high glucose-induced ROS, especially in OA chondrocytes, overcome the inhibitory response induced by TGF, so that MMP-13 gene expression takes longer to be downregulated by TGF when the cells are cultured under high glucose.

These findings are especially important in conditions characterized by abnormal glycemic regulation, namely in diabetes mellitus (DM) patients, where significant variations in glycemia and synovial fluid glucose concentration frequently occur [Brannan and Jerrard, 2006]. In those circumstances, high extracellular glucose concentrations may counteract TGF effects and eventually those of other anabolic growth factors, such as IGF [Kelley et al., 1999], suggesting that hyperglycemia may be an initiating and/or aggravating factor linking DM to OA.

Finally and from another point of view, chondrocytes are frequently exposed in vitro to high glucose concentrations during production of tissue-engineered cartilage. Indeed, it is current practice to use culture media with a high glucose content (4.5 g/L, corresponding to 25 mM) for cartilage tissue engineering [Hennig et al., 2007; Marsano et al., 2007; Das et al., 2008; Appel et al., 2009]. Furthermore, OA cartilage is being increasingly used as a source of chondroprogenitor cells for cartilage tissue engineering [Agar et al., 2011]. Therefore, the results presented in this study warrant caution as to possible deleterious effects of such culture conditions on the chondrogenic differentiation of those cells and on maintenance of the chondrocyte phenotype which directly affect the quality and consequently the in vivo efficacy of the engineered cartilage thus produced.

In conclusion, our study shows that the extracellular glucose concentration has an important role in modulating the expression of genes that are vital for maintenance of the articular cartilage matrix homeostasis. Exposure to high glucose concentrations favors the chondrocyte catabolic gene expression program and may, therefore, promote articular cartilage degradation, facilitating the development and/or progression of OA. Moreover, induction of the chondrocyte catabolic program can also compromise the quality and consequent in vivo efficacy of tissue engineered cartilage.

ACKNOWLEDGMENTS

This work was supported by the Portuguese Foundation for Science and Technology (FCT), grant PTDC/SAU-OSM/67936/2006. SCR and ATR are supported by Ph.D. fellowships (SFRH/BD/19763/2004 and SFRH/BD/47470/2008, respectively) from FCT.

REFERENCES

Agar G, Blumenstein S, Bar-Ziv Y, Kardosh R, Schrift-Tzadok M, Gal-Levy R, Fischler T, Goldschmid R, Yayon A. 2011. The chondrogenic potential of mesenchymal cells and chondrocytes from osteoarthritic subjects. Cartilage 2:40–49.

Andreea SI, Marieta C, Anca D. 2008. AGEs and glucose levels modulate type I and III procollagen mRNA synthesis in dermal fibroblasts cells culture. Exp Diabetes Res 2008:473603.

Appel B, Baumer J, Eyrich D, Sarhan H, Toso S, Englert C, Skodacek D, Ratzinger S, Grässel S, Goepferich A, Blunk T. 2009. Synergistic effects of growth and differentiation factor-5 (GDF-5) and insulin on expanded chondrocytes in a 3-D environment. Osteoarthritis Cartilage 17:1503–1512.

Arner EC. 2002. Aggrecanase-mediated cartilage degradation. Curr Opin Pharmacol 2:322–329.

Baker AH, Edwards DR, Murphy G. 2002. Metalloproteinase inhibitors: Biological actions and therapeutic opportunities. J Cell Sci 115:3719–3727.

Bau B, Gebhard PM, Haag J, Knorr T, Bartnik E, Aigner T. 2002. Relative messenger RNA expression profiling of collagenases and aggrecanases in human articular chondrocytes in vivo and in vitro. Arthritis Rheum 46:2648–2657.

Brannan SR, Jerrard DA. 2006. Synovial fluid analysis. J Emerg Med 30:331–339.

Burner TW, Rosenthal AK. 2009. Diabetes and rheumatic diseases. Curr Opin Rheumatol 21:50–54.

Burrage PS, Mix KS, Brinckerhoff CE. 2006. Matrix metalloproteinases: Role in arthritis. Front Biosci 11:529–543.

Das RHJ, Jahr H, Verhaar JAN, van der Linden JC, van Osch GJVM, Weinans H. 2008. In vitro expansion affects the response of chondrocytes to mechanical stimulation. Osteoarthritis Cartilage 16:385–391.

Death AK, Fisher EJ, McGrath KC, Yue DK. 2003. High glucose alters matrix metalloproteinase expression in two key vascular cells: Potential impact on atherosclerosis in diabetes. Atherosclerosis 168:263–269.

Goldring MB, Marcu KB. 2009. Cartilage homeostasis in health and rheumatic diseases. Arthritis Res Ther 11:224.

Grishko V, Xu M, Ho R, Mates A, Watson S, Kim JT, Wilson GL, Pearsall AW. 2009. Effects of hyaluronic acid on mitochondrial function and mitochondria-driven apoptosis following oxidative stress in human chondrocytes. J Biol Chem 284:9132–9139.

Gurjar MV, Deleon J, Sharma RV, Bhalla RC. 2001. Role of reactive oxygen species in IL-1β-stimulated sustained ERK activation and MMP-9 induction. Am J Physiol 281:H2568–H2574.

Hart DJ, Doyle DV, Spector TD. 1995. Association between metabolic factors and knee osteoarthritis in women: The Chingford Study. J Rheumatol 22:1118–1123.

Hennig T, Lorenz H, Thiel A, Goetzke K, Dickhut A, Geiger F, Richter W. 2007. Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6. J Cell Physiol 211:682–691.

Ho FM, Liu SH, Lin WW, Liau CS. 2007. Opposite effects of high glucose on MMP-2 and TIMP-2 in human endothelial cells. J Cell Biochem 101:442–450.

Ishiguro N, Ito T, Ito H, Iwata H, Jugessur H, Ionescu M, Poole AR. 1999. Relationship of matrix metalloproteinases and their inhibitors to cartilage proteoglycan and collagen turnover: Analyses of synovial fluid from patients with osteoarthritis. Arthritis Rheum 42:129–136.

Kelley KM, Johnson TR, Ilan J, Moskowitz RW. 1999. Glucose regulation of the IGF response system in chondrocytes: Induction of an IGF-I-resistant state. Am J Physiol 276:R1164–R1171.

Kevorkian L, Young DA, Darrah C, Donell ST, Shepstone L, Porter S, Brockbank SM, Edwards DR, Parker AE, Clark IM. 2004. Expression profiling of metalloproteinases and their inhibitors in cartilage. Arthritis Rheum 50:131–141.

Kim J-J, Li J-J, Kim KS, Kwak S-J, Jung D-S, Ryu D-R, Yoo T-H, Choi HY, Han SH, Kim HJ, Yoon SY, Han DS, Kang S-W. 2008. High glucose decreases

collagenase expression and increases TIMP expression in cultured human peritoneal mesothelial cells. Nephrol Dial Transplant 23:534–541.

Lo YY, Conquer JA, Grinstein S, Cruz TF. 1998. Interleukin-1 beta induction of c-fos and collagenase expression in articular chondrocytes: Involvement of reactive oxygen species. J Cell Biochem 69:19–29.

Maneiro E, Martin MA, de Andres MC, Lopez-Armada MJ, Fernandez-Sueiro JL, del Hoyo P, Galdo F, Arenas J, Blanco FJ. 2003. Mitochondrial respiratory activity is altered in osteoarthritic human articular chondrocytes. Arthritis Rheum 48:700–708.

Marlovits S, Hombauer M, Truppe M, Vecsei V, Schlegel W. 2004. Changes in the ratio of type-I and type-II collagen expression during monolayer culture of human chondrocytes. J Bone Joint Surg Br 86:286–295.

Marsano A, Millward-Sadler SJ, Salter DM, Adesida A, Hardingham T, Tognana E, Kon E, Chiari-Grisar C, Nehrer S, Jakob M, Martin I. 2007. Differential cartilaginous tissue formation by human synovial membrane, fat pad, meniscus cells and articular chondrocytes. Osteoarthritis Cartilage 15:48–58.

Martel-Pelletier J, Boileau C, Pelletier JP, Roughley PJ. 2008. Cartilage in normal and osteoarthritis conditions. Best Pract Res Clin Rheumatol 22:351–384.

Martin I, Jakob M, Schafer D, Dick W, Spagnoli G, Heberer M. 2001. Quantitative analysis of gene expression in human articular cartilage from normal and osteoarthritic joints. Osteoarthritis Cartilage 9:112–118.

McNulty AL, Stabler TV, Vail TP, McDaniel GE, Kraus VB. 2005. Dehydroascorbate transport in human chondrocytes is regulated by hypoxia and is a physiologically relevant source of ascorbic acid in the joint. Arthritis Rheum 52:2676–2685.

Mendes AF, Caramona MM, Carvalho AP, Lopes MC. 2003a. Differential roles of hydrogen peroxide and superoxide in mediating IL-1-induced NF-kappa B activation and iNOS expression in bovine articular chondrocytes. J Cell Biochem 88:783–793.

Mendes AF, Caramona MM, Carvalho AP, Lopes MC. 2003b. Hydrogen peroxide mediates interleukin-1beta-induced AP-1 activation in articular chondrocytes: Implications for the regulation of iNOS expression. Cell Biol Toxicol 19:203–214.

Miosge N, Hartmann M, Maelicke C, Herken R. 2004. Expression of collagen type I and type II in consecutive stages of human osteoarthritis. Histochem Cell Biol 122:229–236.

Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA, Rosner PJ, Geoghegan KF, Hambor JE. 1996. Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. J Clin Invest 97:761–768.

Miyazaki Y, Tsukazaki T, Hirota Y, Yonekura A, Osaki M, Shindo H, Yamashita S. 2000. Dexamethasone inhibition of TGF beta-induced cell growth and type II collagen mRNA expression through ERK-integrated AP-1 activity in cultured rat articular chondrocytes. Osteoarthritis Cartilage 8: 378–385.

Nagase H, Kashiwagi M. 2003. Aggrecanases and cartilage matrix degradation. Arthritis Res Ther 5:94–103.

Pallu S, Francin P-J, Guillaume C, Gegout-Pottie P, Netter P, Mainard D, Terlain B, Presle N. 2010. Obesity affects the chondrocyte responsiveness to leptin in patients with osteoarthritis. Arthritis Res Ther 12:R112.

Richardson S, Neama G, Phillips T, Bell S, Carter SD, Moley KH, Moley JF, Vannucci SJ, Mobasheri A. 2003. Molecular characterization and partial cDNA cloning of facilitative glucose transporters expressed in human articular chondrocytes; stimulation of 2-deoxyglucose uptake by IGF-I and elevated MMP-2 secretion by glucose deprivation. Osteoarthritis Cartilage 11:92–101.

Rosa SC, Goncalves J, Judas F, Mobasheri A, Lopes C, Mendes AF. 2009. Impaired glucose transporter-1 degradation and increased glucose transport and oxidative stress in response to high glucose in chondrocytes from osteoarthritic versus normal human cartilage. Arthritis Res Ther 11:R80.

Seifarth C, Csaki C, Shakibaei M. 2009. Anabolic actions of IGF-I and TGF-beta1 on Interleukin-1beta-treated human articular chondrocytes: Evaluation in two and three dimensional cultures. Histol Histopathol 24:1245–1262.

Siviero P, Tonin P, Maggi S. 2009. Functional limitations of upper limbs in older diabetic individuals. The Italian longitudinal study on aging. Aging Clin Exp Res 21:458–462.

Sturmer T, Brenner H, Brenner RE, Gunther KP. 2001. Non-insulin dependent diabetes mellitus (NIDDM) and patterns of osteoarthritis. The Ulm osteoarthritis study. Scand J Rheumatol 30:169–171.

Su S, Grover J, Roughley PJ, DiBattista JA, Martel-Pelletier J, Pelletier JP, Zafarullah M. 1999. Expression of the tissue inhibitor of metalloproteinases (TIMP) gene family in normal and osteoarthritic joints. Rheumatol Int 18: 183–191.

Takaishi H, Kimura T, Dalal S, Okada Y, D'Armiento J. 2008. Joint diseases and matrix metalloproteinases: A role for MMP-13. Curr Pharm Biotechnol 9:47–54.

Tesche F, Miosge N. 2005. New aspects of the pathogenesis of osteoarthritis: The role of fibroblast-like chondrocytes in late stages of the disease. Histol Histopathol 20:329–337.

Velasquez MT, Katz JD. 2010. Osteoarthritis: Another component of metabolic syndrome? Metab Syndr Relat Disord 8:295–305.

Wang L, Almqvist KF, Veys EM, Verbruggen G. 2002. Control of extracellular matrix homeostasis of normal cartilage by a TGFbeta autocrine pathway. Validation of flow cytometry as a tool to study chondrocyte metabolism in vitro. Osteoarthritis Cartilage 10:188–198.

Zafarullah M, Su S, Martel-Pelletier J, DiBattista JA, Costello BG, Stetler-Stevenson WG, Pelletier JP. 1996. Tissue inhibitor of metalloproteinase-2 (TIMP-2) mRNA is constitutively expressed in bovine, human normal, and osteoarthritic articular chondrocytes. J Cell Biochem 60:211–217.