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Comparison between chondroprotective effects of glucosamine, curcumin, and diacerein in IL-1β-stimulated C-28/I2 chondrocytes

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Summary

Objective: To compare the effects of glucosamine (GlcN), curcumin, and diacerein in immortalized human C-28/I2 chondrocytes at the cellular and the gene expression level. This study aimed to provide insights into the proposed beneficial effects of these agents and to assess the applicability of the C-28/I2 cell line as a model for the evaluation of chondroprotective action.

Methods: Interleukin-1beta (IL-1β)-stimulated C-28/I2 cells were cultured in the presence of GlcN, curcumin, and diacerein prior to the evaluation of parameters such as viability, morphology and proliferation. The impact of GlcN, curcumin, and diacerein on gene expression was determined using quantitative real-time RT-PCR (qPCR).

Results: At the transcriptional level, 5 mM GlcN and 50 μM diacerein increased the expression of cartilage-specific genes such as aggrecan (AGC) and collagen type II (COL2), while reducing collagen type I (COL1) mRNA levels. Moreover, the IL-1β-mediated shift in gene expression pattern was antagonized by GlcN and diacerein. These effects were associated with a significant reduction in cellular proliferation and the development of chondrocyte-specific cell morphology. In contrast, curcumin was not effective at lower concentrations but even damaged the cells at higher amounts.

Conclusions: Both GlcN and diacerein promoted a differentiated chondrocytic phenotype of immortalized human C-28/I2 chondrocytes by altering proliferation, morphology, and COL2/COL1 mRNA ratios. Moreover, both agents antagonized inhibitory effects of IL-1β by enhancing AGC and COL2 as well as by reducing COL1 mRNA levels.

Key words: Chondrocytes, Glucosamine, Curcumin, Diacerein, Chondroprotection, qPCR.

Introduction

Osteoarthritis (OA) is a chronic, degenerative joint disorder characterized by enhanced degradation of major extracellular matrix (ECM) components as well as by extensive changes in the types and amounts of newly synthesized ECM molecules[1,2]. To date, OA represents a non-curable disease and classic therapeutic approaches are limited to symptom-relieving drugs or surgical intervention. Chondroprotection with drugs possessing disease-modifying qualities represents an alternative concept in the treatment of OA aiming to intervene therapeutically in the course of cartilage degradation with pharmacological agents[3]. Glucosamine (GlcN), curcumin, and diacerein are examples of such chondroprotective agents (CPA). Studies performed both *in vitro* and *in vivo*, however, have yielded partially inconsistent findings[4–12]. Although medical opinion about the applicability and clinical efficacy of CPA in OA remains controversial, these agents are widely available and generally well tolerated and may possess more desirable safety profiles as compared to non-steroidal anti-inflammatory drugs (NSAIDs). A detailed clarification of the molecular effects of CPA is therefore regarded as important for our understanding of OA modifying action.

For the investigation of chondroprotective action, interleukin-1beta (IL-1β)-stimulated chondrocyte models represent a helpful tool[5,7,8,12–14]. In these models, cultured chondrocytes are incubated with both an inflammatory mediator such as IL-1β and the CPA of interest, prior to the investigation of selected targets at the protein or gene expression level. However, it is well known that studies using primary cultures of human articular chondrocytes suffer from numerous limitations such as poor yields, little proliferative activity, and inter-individual variability of donor samples. In order to circumvent these drawbacks, the human chondrocyte cell line C-28/I2 was established by retroviral-mediated transfection of primary rib chondrocytes with the large T antigen

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of Simian Virus 40[15]. C-28/I2 cells have been shown to retain chondrocytic morphology, to maintain continuous proliferation in monolayer culture and to express well-differentiated chondrocyte-specific genes, particularly matrix-anabolic and matrix-catabolic genes. In recent studies, C-28/I2 cells have been used to investigate chondrocytic response to IL-1β, nitric oxide, hyaluronan oligosaccharides, or oxygen species[16–18]. To date, only one study has been published that reported the application of C-28/I2 cells for the evaluation of chondroprotective effects using extracts of *Schisandra fructus*[19]. Therein, Choi *et al.* observed protective effects of herbal components on aggrecan (AGC) expression against hyaluronidase treatment.

From this background, the present study aimed to compare the chondroprotective activities of GlcN, curcumin, and diacerein in immortalized human chondrocytes. IL-1β-challenged C-28/I2 chondrocytes were cultured in the presence of GlcN, curcumin, or diacerein and parameters such as viability, morphology, and proliferation were assessed. In addition, mRNA levels of both anabolic and catabolic marker genes were determined using quantitative real-time RT-PCR (qPCR). Our results will provide insights into the proposed beneficial effects of GlcN, curcumin, and diacerein and information on the applicability of IL-1β-stimulated C-28/I2 cells as a model for evaluating chondroprotective action.

Materials and methods

MATERIALS

GlcN, curcumin, diacerein, trypsin-ethylene diamine tetraacetic acid (EDTA), fluorescein diacetate (FDA) and ethidium bromide (EB) were purchased from Sigma (St. Louis, MO, USA). Recombinant human IL-1β was obtained from Strathmann (Hamburg, D). qPCR primers were from Metabion (Martinsried, D). Dulbecco's modified Eagle's medium (DMEM) and insulin-transferrin-selenium-solution (ITS) were purchased from Gibco (Lofer, A). The StrataScript First-Strand Synthesis System and the Brilliant SYBR Green QPCR Master Mix were from Stratagene (La Jolla, CA, USA).

CELL CULTURE

Immortalized C-28/I2 chondrocytes, previously described by Goldring *et al.*[15], were used throughout the study. Cells were seeded at 9.3×10^3 cells per cm^2 and cultured using DMEM supplemented with 10% fetal calf serum (Biochrom AG, Berlin, D), 2 μl/ml gentamycin, and 50 μg/ml ascorbate as culture medium. Upon confluence, cells were split using 0.03% trypsin-EDTA and seeded at the same density for viability, morphology, proliferation, and qPCR assays. GlcN was dissolved in culture medium, whereas curcumin and diacerein were dissolved in 96% ethanol and >99.9% dimethyl sulfoxide (DMSO), respectively, prior to addition to the medium.

CELL VIABILITY AND CELL MORPHOLOGY

Chondrocytes were cultured to confluence on glass cover slips. The confluent stage was selected to provide a reproducible stage of cell culture for all assays. Cell monolayers were challenged with either 10 ng/ml IL-1β, 5 mM GlcN, 5 μM curcumin, 50 μM curcumin, or 50 μM diacerein. Untreated cells served as control representing viable cells. After 24 h, the cell culture medium was removed and 400 μl dying solution containing 8 μg/ml FDA and 10 μg/ml EB in PBS were added to each well. After 5 min, cells were inspected using a Nikon Eclipse 50i microscope equipped with an EXFO X-Cite 120 fluorescence illumination system. Using the ImageJ 1.36b software, the percentage of EB-stained cells was calculated with respect to methanol (MeOH)-treated cells (50 μl MeOH; 5 min; −20°C) representing 100% dead cells.

In addition, morphological alterations of C-28/I2 cells upon treatment with 10 ng/ml IL-1β, 5 mM GlcN, 5 μM curcumin, and 50 μM diacerein were observed using light microscopy.

PROLIFERATION ASSAY

Chondrocytes were grown to confluence in 96-well microplates (Iwaki, Tokyo, Japan). Then, culture medium supplemented with either 10 ng/ml IL-1β, 10 ng/ml IL-1β and 5 mM GlcN, 10 ng/ml IL-1β and 5 μM curcumin, or 10 ng/ml IL-1β and 50 μM diacerein was added. Cultures without IL-1β and CPA were used as controls. After 24 h, the quantity of total cells was determined using the CyQuant assay (Molecular Probes, Eugene, OR, USA) pursuing a modified protocol[20]. The rate of proliferating cells was assessed by incorporation of 2-bromodesoxyuridine into the DNA of dividing cells using the colorimetric BrdU assay (Roche, Mannheim, Germany).

EXPERIMENTAL CONDITIONS FOR qPCR

On days 5 and 6 after seeding into 25 cm^2 culture flasks (Corning, NY, USA), cells were exposed to serum-free ITS medium supplemented with the particular CPA (GlcN, curcumin, or diacerein) and/or IL-1β (10 ng/ml) as indicated in Table I. Seven conditions for each CPA experiment were created: one control group without additional supplements, one IL-1β treated group, two groups simultaneously treated with both IL-1β and the CPA at two different concentrations, two groups pre-incubated with the CPA at two different concentrations prior to IL-1β addition, and one group treated with the respective CPA at the higher concentration without the addition of IL-1β. Each of these study groups consisted of three identically treated biological replicates.

On day 7 after seeding, total RNA was extracted using the NucleoSpin RNA II Kit (Macherey-Nagel, Dueren, D). All RNA samples were qualitatively assessed on agarose gels and quantified using the Quant-It RiboGreen reagent (Molecular Probes, Eugene, OR, USA) and the Stratagene MxPro QPCR software. Reverse transcription (RT) into cDNA was performed using the StrataScript First-Strand Synthesis System. Briefly, 2 μg of total RNA were reverse transcribed in a 20 μl reaction volume using oligo(dT) primers. All cDNA preparations were diluted at a ratio of 1:10 with RNase free water prior to qPCR.

OPTIMIZATION OF qPCR ASSAYS

Primers for AGC, collagen type II (COL2) and collagen type I (COL1), matrix metalloproteinase 3 (MMP3), "A distingegrin and metalloproteinases with thrombospondin type 1 motif" (ADAMTS4) and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were designed using the AlleleID 2.01 software (Premier Biosoft, CA, USA). Sequences are listed in Table II. Primers for beta-2-microglobulin (B2M) and hypoxanthine phosphoribosyl-transferase I (HPRT1) were used as previously described[21,22].

Primer concentrations for the qPCR experiments were optimized using a matrix of forward and reverse primers varying in concentration from 50 to 900 nM and a purified PCR product as template[21]. Amplification efficiencies for each primer pair were derived from standard curves using dilution series of a control cDNA sample. Both optimized primer concentrations and amplification efficiencies are listed in Table II.

qPCR

All qPCR reactions were performed in 25 μl reaction mixtures containing 1 μl cDNA, 12.5 μl Brilliant SYBR Green qPCR Master Mix, primer pairs as listed in Table II, and nuclease-free water to 25 μl. Each biological replicate was run in duplicate on an Mx3000P qPCR system. Thermocycling

Table I
Experimental conditions for qPCR experiments. Medium supplements were added to the chondrocyte culture medium at the following concentrations: IL-1β, 10 ng/ml recombinant human IL-1β; [c]1, low concentration of GlcN (50 μM), curcumin (5 μM) or diacerein (5 μM); [c]2, high concentration of GlcN (5 mM), curcumin (50 μM) or diacerein (50 μM)

Experimental condition	Supplements on days of culture	
	Day 5	Day 6
Control	–	–
IL-1β	–	IL-1β
Simultaneous incubation with CPA [c]1	–	[c]1 + IL-1β
Simultaneous incubation with CPA [c]2	–	[c]2 + IL-1β
Pre-incubation with CPA [c]1	[c]1	[c]1 + IL-1β
Pre-incubation with CPA [c]2	[c]2	[c]2 + IL-1β
CPA only	[c]2	[c]2

Table II
Sequences, optimal concentrations and amplification efficiencies of qPCR primers. In order to avoid non-specific product formation, primers were designed to span introns and with minimized self and cross dimer ΔG values. Additionally, BLAST analyses were performed to verify specificity

Gene symbol	Primer sequences	nM	Efficiency (%)
AGC	Fw: ACTGGCGAGCACTGTAAC	300	98.6
	Rv: TCTTGGGCATTGTTGTTGAC	900	
COL2	Fw: TGGTGGAGCAGCAAG	600	92.4
	Rv: GGGAGGCGTGAGGTCTTCTG	600	
COL1	Fw: CACTGGTGATGCTGGTCCTG	600	89.2
	Rv: CGAGGTCACGGTCACGAAC	300	
MMP3	Fw: GGTGTGGAGTTCCTGATGTTG	100	99.9
	Rv: AGCCTGGAGAATGTGAGTGG	100	
ADAMTS4	Fw: ACCCAAGCATCCGCAATCC	100	90.1
	Rv: GCCCACATCAGCCATACCC	100	
GAPDH	Fw: GGAGTCCACTGGCGTCTTCAC	300	94.0
	Rv: GAGGCATTGCTGATGATCTTGAGG	600	

conditions for AGC, COL1, MMP3, and ADAMTS4 consisted of an initial polymerase activation step at 95°C for 10 min, followed by 45 cycles at 95°C for 30 s, at 55°C for 1 min, and at 72°C for 1 min. Thermocycling conditions for COL2 were optimized and consisted of an initial polymerase activation step at 95°C for 10 min, followed by 45 cycles at 95°C for 30 s, at 58°C for 45 s, and at 80°C for 1 min. Afterwards, melting curves were generated to confirm a single gene-specific peak and to detect primer–dimer formation. No-template controls were included in each run to control for contaminations.

DATA NORMALIZATION AND STATISTICS

Based on our previous report addressing the importance of reference gene selection in qPCR, gene expression data of AGC, COL1, and COL2 were normalized against GAPDH as single reference gene[21]. In order to visualize up- and down-regulation of ECM-related genes in the figures more clearly, expression levels were log-transformed (log base of 2) and alterations with respect to the control group were displayed as log-fold changes. Consequently, a log-fold change of 1 in the figures corresponds to a two-fold alteration in relative quantity of mRNA levels. In contrast, a log-fold change of −1 corresponds to a 0.5-fold alteration in relative quantity (equivalent to a 50% reduction). Ratios of mRNA levels of COL2 to COL1 (COL2/COL1) were calculated as 'differentiation index' according to Ref. 2. Data of the MMP3 and ADAMTS4 experiments were normalized using normalization factors derived from the geometric mean of GAPDH, B2M, and HPRT1 and the geNorm software[21,22]. mRNA levels of MMP3 and ADAMTS4 in the samples were displayed as relative quantities with respect to the untreated control group. Statistics were performed using one-way analysis of variance (ANOVA) with post-hoc Tukey tests cross-comparing all study groups ($P < 0.05$). Statistics of the viability and proliferation data were performed using the Student's t test ($P < 0.05$).

Results

CELL VIABILITY AND MORPHOLOGY

Figure 1(A) shows the viability of chondrocytes in response to CPA treatment. Untreated control cells hydrolyzed the FDA reagent resulting in an intense green staining (Fig. 1-A1). Similar results were obtained after exposure of cells to 10 ng/ml IL-1β, 5 mM GlcN, 50 μM diacerein, or 5 μM curcumin (Fig. 1-A2). Whereas all other culture conditions evoked minor staining with EB indicating a high rate of viability (Fig. 1-A3), incubation of chondrocytes with 50 μM curcumin resulted in intensive red staining of cell nuclei with EB (Fig. 1-A4) indicating the presence of damaged cells. Moreover, those cells became partly detached from the cover slips. Graphical evaluation of cellular viability yielded an area of dead cells of 59.2 ± 3.4% in case of MeOH-treated cells (representing 100% dead cells). Compared to the untreated control prepared with PBS

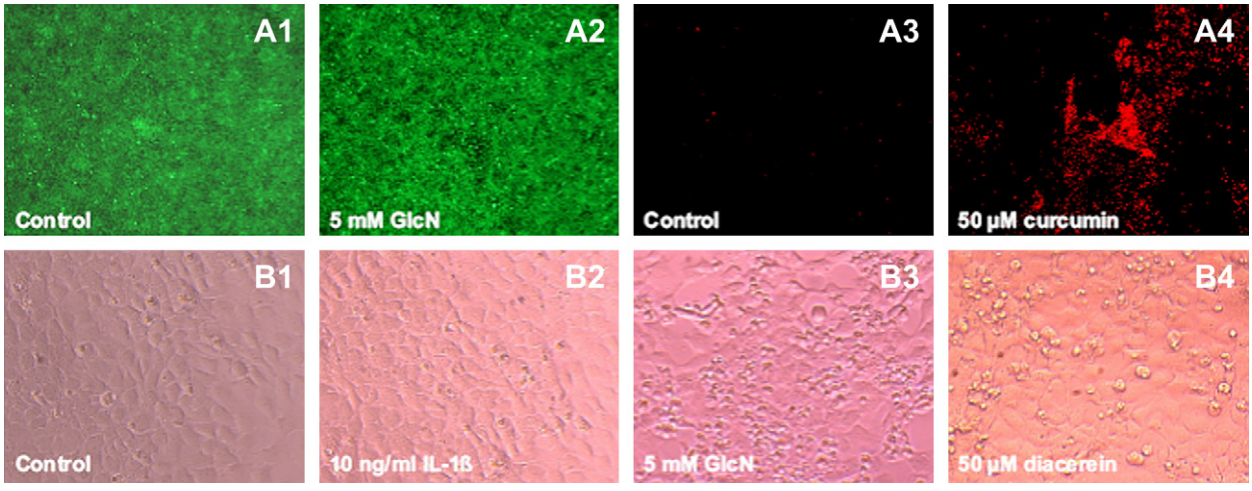

Fig. 1. (A) Viability of C-28/I2 chondrocytes in response to CPA. Viable cells appear green due to hydrolysis of the FDA reagent (A1, A2) whereas EB-stained red cell nuclei indicate damaged cells (A3, A4). Quantitative results of the image analysis are described in the Results. (B) Morphological changes of C-28/I2 chondrocytes in response to CPA or IL-1β. The substances were added to confluent cell monolayers and photographs were obtained *via* optical microscope after 48 h exposure.

(4.6 ± 1.1%), incubation with 10 ng/ml IL-1β, 5 mM GlcN, 50 μM diacerein, or 5 μM curcumin yielded no significant differences (<6.5% dead cells). In contrast, treatment of chondrocytes with 50 μM curcumin resulted in 42.9 ± 8.4% dead cells.

Figure 1(B) shows the cell morphology of C-28/I2 chondrocytes under IL-1β or CPA exposure. Without CPA or under treatment with IL-1β the cells of the confluent monolayers had a polygonal morphology (Fig. 1-B1 and B2). No alteration was observed after treatment with 5 μM curcumin (not shown). However, upon incubation with 5 mM GlcN or 50 μM diacerein a larger fraction of cells was characterized by the chondrocyte-specific 'spherical' morphology (Fig. 1-B3 and B4).

PROLIFERATION

The results of the CyQuant and the BrdU assays are presented in Fig. 2 as percentages of either total cell count or the number of proliferating cells with respect to the untreated control representing 100%. The results demonstrate that neither of the treatments had a significant impact on the total number of cells. Regarding the proliferative activity, neither IL-1β nor 5 μM curcumin significantly influenced this parameter. The absent effect of IL-1β on C-28/I2 cell proliferation is contradictory to the behavior of primary chondrocytes and might represent a special feature of C-28/I2 chondrocytes, presumably attributed to their immortalized character. Interestingly, the addition of 5 mM GlcN or 50 μM diacerein resulted in a significant 20.6% and 14.1% decrease of proliferating cells, respectively.

EFFECTS OF IL-1β AT THE mRNA LEVEL

The effects of IL-1β on the expression of ECM-associated genes are depicted in Figs. 3–5 representing three independent experiments. A high concentration of IL-1β (10 ng/ml) was used to assure a maximal osteoarthritic effect in chondrocytes *in vitro*[23]. Incubation of C-28/I2 cells with IL-1β for 24 h resulted in 0.75-fold (−0.41-log fold; Fig. 3), 0.57-fold (−0.82-log fold; Fig. 4), and 0.63-fold (−0.66-log fold; Fig. 5) decreased AGC mRNA levels. Significant suppression of COL2 mRNA levels was observed in all experiments [from 0.68-fold (−0.54-log fold; Fig. 3) to 0.49-fold (−1.01-log fold; Fig. 5)]. In contrast, levels of COL1 were up-regulated 1.55-fold (0.64-log fold), 1.51-fold (0.60-log fold) and 1.68-fold (0.75-log fold) in the three experiments, respectively. Regarding catabolic marker genes, we found that IL-1β did not induce any significant change in either MMP3 or ADAMTS4 expression in C-28/I2 chondrocytes (Table III).

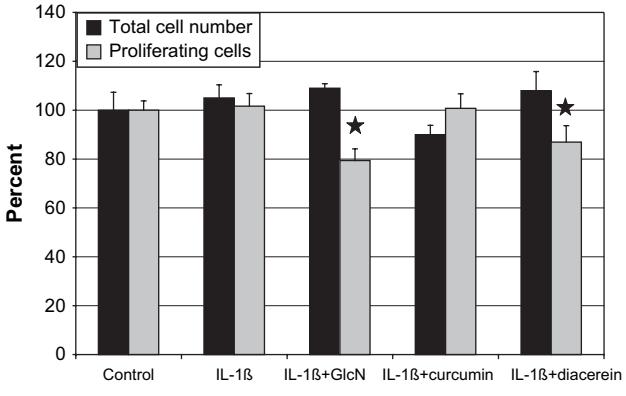

Fig. 2. Total cell count and proliferative activity of C-28/I2 cells after incubation with IL-1β and/or 5 mM GlcN, 5 μM curcumin, or 50 μM diacerein. The number of total cells and proliferating cells was determined using CyQuant and BrdU assays, respectively. Values of the untreated control group were set to 100% and the influence of IL-1β, GlcN, curcumin, and diacerein was calculated with regard to this benchmark. Significant differences as compared to the untreated control are marked with asterisks ($P < 0.05$).

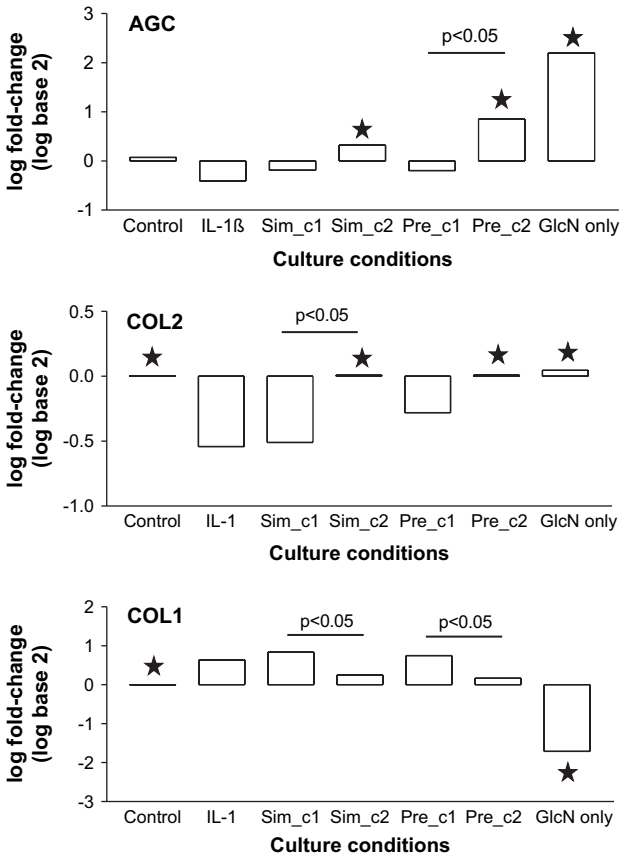

Fig. 3. Regulation of AGC, COL2, and COL1 mRNA expression by IL-1β and/or GlcN added to C-28/I2 chondrocytes under various culture conditions (Table I). Significant differences as compared to the IL-1β treated group are marked with asterisks ($P < 0.05$). Significant dose-related differences between study groups (Table I) are marked with brackets.

EFFECTS OF GlcN AT THE mRNA LEVEL

Figure 3 shows the influence of GlcN on mRNA levels of AGC, COL2, and COL1 in C-28/I2 chondrocytes. The graphs visualize that the incubation of chondrocytes with 5 mM GlcN for 48 h without adding IL-1β ('GlcN only') induced contrary effects to those of IL-1β in case of AGC and COL1. While AGC mRNA levels were up-regulated 4.6-fold (2.2-log fold) by GlcN, COL1 expression was decreased to a significant extent (0.31-fold; −1.71-log fold). In contrast, 5 mM GlcN did not alter COL2 expression.

Of note, GlcN was found to antagonize the effects of IL-1β in a dose-dependent manner. In this context, the inhibitory effect of IL-1β on AGC was reversed by 5 mM

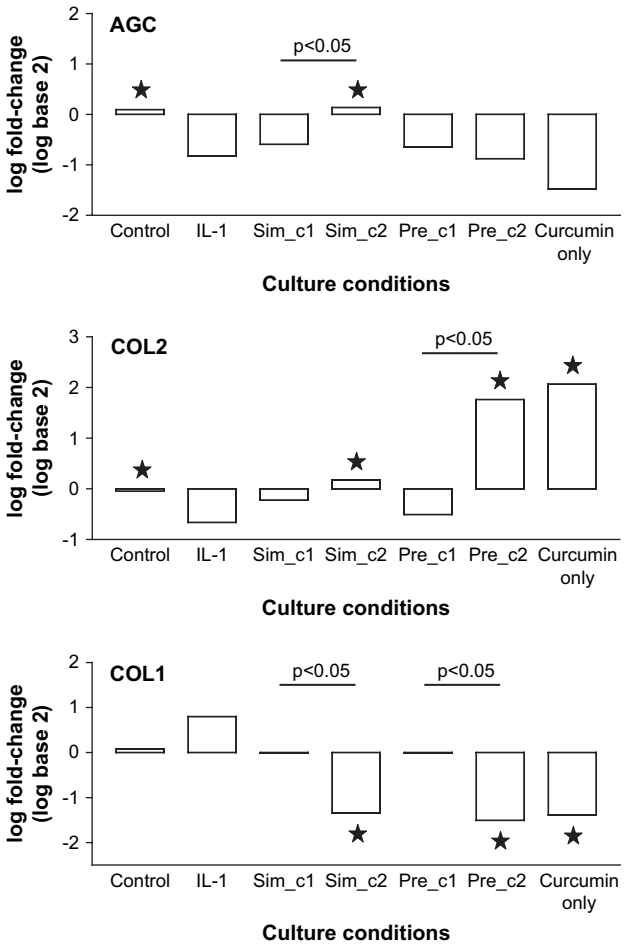

Fig. 4. Regulation of AGC, COL2, and COL1 mRNA expression by IL-1β and/or curcumin added to C-28/I2 chondrocytes under various culture conditions (Table I). Significant differences as compared to the IL-1β treated group are marked with asterisks ($P < 0.05$). Significant dose-related differences between study groups (Table I) are marked with brackets.

GlcN in both the simultaneous-addition ('sim_c2') and pre-incubation ('pre_c2') experiments resulting in a 1.25-fold (0.32-log fold) and 1.8-fold (0.85-log fold) AGC up-regulation, respectively. Regarding COL2 mRNA expression, addition of 5 mM GlcN ('sim_c2' and 'pre_c2') reversed the action of IL-1β and increased COL2 levels to values comparable to those of the untreated control. Moreover, a comparison between 50 μM and 5 mM GlcN revealed a significant and dose-dependent down-regulation of COL1 in both the simultaneous-addition and pre-incubation experiments.

As shown in Table III GlcN did not regulate the transcription of MMP3 and ADAMTS4 to a significant extent.

EFFECTS OF CURCUMIN AT THE mRNA LEVEL

The influence of curcumin on ECM-related genes in C-28/I2 cells is presented in Fig. 4. Curcumin (5 μM) – a concentration that did not affect viability or proliferation of C-28/I2 cells – was not effective in preventing IL-1β-mediated down-regulation of AGC or COL2 in either the simultaneous-addition ('sim_c1') or the pre-incubation experiments ('pre_c1').

Despite the damaging effects of 50 μM curcumin on C-28/I2 cell viability (Fig. 1), we were interested in how this process might be reflected at the level of gene expression. Interestingly, incubation of chondrocytes with 50 μM curcumin for 48 h ('curcumin only') provoked a 4.2-fold (2.07-log fold) increase of COL2 and a 0.38-fold (−1.39-log fold) decrease in COL1 expression as compared to the untreated control group. In addition, the presence of 50 μM curcumin remarkably increased the expression of both MMP3 (2175.5-fold) and ADAMTS4 (119.7-fold) (Table III).

EFFECTS OF DIACEREIN AT THE mRNA LEVEL

The supporting effect of diacerein on chondrocytic differentiation was demonstrated by 3.19-fold (1.16-log fold) and 1.84-fold (0.61-log fold) increased levels of AGC and COL2 expression, respectively, when cells were exposed to 50 μM diacerein for 48 h ('diacerein only') (Fig. 5). Under the same conditions, transcription of COL1 was down-regulated 0.13-fold (−2.89-log fold).

Significant dose-dependent effects of diacerein on AGC and COL1 mRNA levels were observed when cells were additionally exposed to IL-1β. Incubation of cells with 50 μM diacerein ('c2') reversed the IL-1β-mediated modulations of AGC and COL1 in both the simultaneous-addition and pre-incubation experiments, whereas 5 μM ('c1') did not. However, 5 μM diacerein was effective in reducing the cytokine-induced down-regulation of the COL2 transcription.

Regarding its impact on catabolic marker genes, we found that diacerein did not alter MMP3 and ADAMTS4 mRNA levels significantly as compared to untreated controls (Table III).

Discussion

GlcN AND DIACEREIN PROMOTE THE CHONDROCYTIC PHENOTYPE OF C-28/I2 CELLS

In their natural surroundings chondrocytes exhibit low mitotic activity and maintain the cartilage function by synthesis of ECM-related proteins such as COL2 or AGC. During *in vitro* expansion in monolayers, however, primary chondrocytes have the tendency to dedifferentiate towards a fibroblast-like phenotype. Several studies have reported that certain culture conditions can promote expansion of primary chondrocytes but concomitantly induce dedifferentiation[24–28]. The process of dedifferentiation is further associated with modifications of the gene expression pattern such as the decrease of COL2/COL1 mRNA ratios and the loss of the round cell shape[29]. To overcome this problem, various culture models have been developed including cell pellet cultures or embedding in alginate beads[10,24,30,31]. Although the use of immortalized chondrocyte cell lines in cartilage research has many advantages, the culture of immortalized C-28/I2 cells in alginate beads is limited as the proliferating cells form necrotic clusters with reduced cytoplasm and die.

Here, we demonstrated that GlcN and diacerein can promote chondrocyte-specific characteristics of immortalized C-28/I2 chondrocytes. The addition of 5 mM GlcN or 50 μM diacerein to the culture medium significantly reduced the number of dividing cells. Viability, however, was not affected. This process was further accompanied by the formation of a 'spherical' cell morphology which is known to be chondrocyte-specific[32]. In contrast, incubation

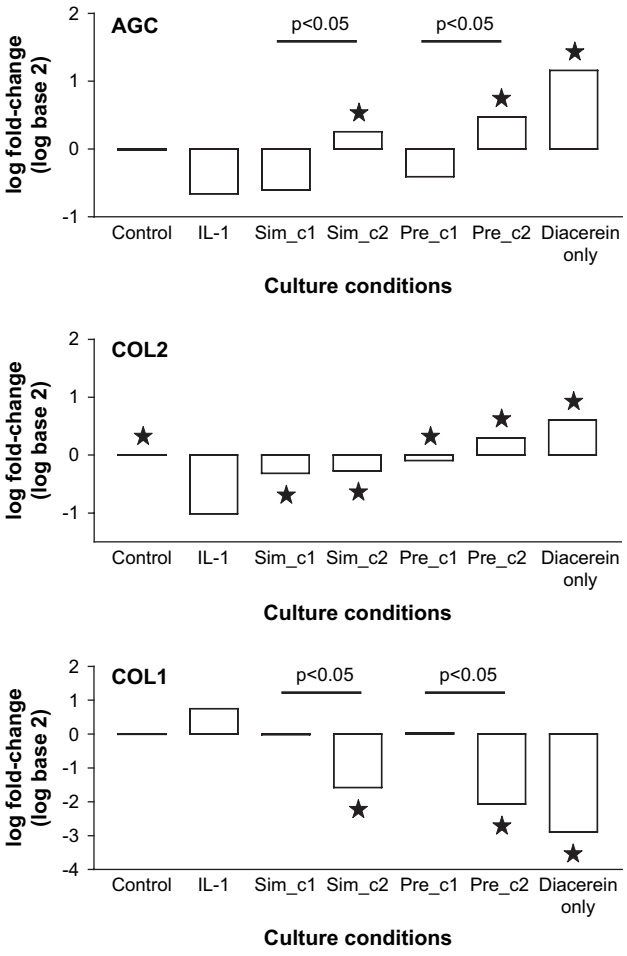

Fig. 5. Regulation of AGC, COL2, and COL1 mRNA expression by IL-1β and/or diacerein added to C-28/I2 chondrocytes under various culture conditions (Table I). Significant differences as compared to the IL-1β treated group are marked with asterisks ($P < 0.05$). Significant dose-related differences between study groups (Table I) are marked with brackets.

of cells with 5 μM curcumin neither altered significantly the rate of proliferation nor modulated the cell morphology. These results are in agreement with our findings on gene expression. Using qPCR, both GlcN and diacerein were shown to be effective in promoting the mRNA expression of the chondrocytic marker AGC. However, GlcN – at a concentration (5 mM) being 100-fold higher than diacerein (50 μM) – induced a 44% higher up-regulation of AGC as compared to diacerein (4.6-fold vs 3.19-fold up-regulation). Moreover, COL2/COL1 ratios – previously defined as index of cell differentiation[2] – were determined. Whereas 5 μM curcumin did not influence the COL2/COL1 ratio significantly, the treatment with 5 mM GlcN resulted in 4.5-fold increased values (0.18 ± 0.03; $P < 0.05$) as compared to the control samples (0.04 ± 0.02). In comparison, the presence of 50 μM diacerein increased the COL2/COL1 ratio in C-28/I2 chondrocytes 9.5-fold (from $0.0008 \pm 8.1 \times 10^{-5}$ in the control samples to 0.0076 ± 0.0011 in the challenged samples; $P < 0.05$).

All in all, our findings suggest that GlcN and diacerein can induce a shift of immortalized C-28/I2 cells towards a more chondrocytic phenotype.

GlcN AND DIACEREIN ANTAGONIZE IL-1β-MEDIATED EFFECTS IN C-28/I2 CELLS

It is well known that cytokines such as tumor necrosis factor-alpha (TNF-α) or IL-1β contribute to the progress of cartilage degradation in OA[1]. In the present study, we have investigated and compared the ability of GlcN, curcumin, and diacerein to antagonize the action of IL-1β in C-28/I2 chondrocytes at the mRNA level. When added to the culture medium simultaneously with IL-1β, both GlcN and diacerein dose-dependently reduced the impact of the cytokine. At lower concentrations neither agent was chondroprotective on COL1 or AGC mRNA expression. Regarding COL2 transcription, however, 5 μM diacerein evoked a significant up-regulation whereas 50 μM GlcN were ineffective. At higher concentrations (5 mM GlcN and 50 μM diacerein) both substances significantly antagonized the effects of IL-1β. When added simultaneously with IL-1β, 5 mM GlcN increased AGC levels by 78% as compared to IL-1β treated cells, whereas 50 μM diacerein induced an enhancement by 87%. Regarding COL2 transcription, 5 mM GlcN and 50 μM diacerein induced 37% and 29% higher expression levels, respectively. Moreover, IL-1β-stimulated COL1 levels were reduced by 84% when cells were exposed to 50 μM diacerein whereas there was no significant reduction in presence of 5 mM GlcN.

Taken together, our findings suggest that both GlcN and diacerein can prevent or reduce the IL-1β-induced inhibition of ECM synthesis. However, diacerein appeared to be more effective than GlcN since comparable or even better results were obtained at much lower concentrations (5 mM GlcN vs 50 μM diacerein). This difference might be attributed to different mechanisms of action[12,33] and might be of interest regarding the bioavailability of these agents after oral administration.

COMPARISON BETWEEN CHONDROPROTECTIVE EFFECTS IN C-28/I2 CELLS AND PRIMARY CHONDROCYTES

A further aim of this study was to evaluate the applicability of the C-28/I2 cell line as a model for the investigation of chondroprotective effects. Therefore, the findings of the present report were compared to those of studies on primary human chondrocytes in the literature. Indeed, our results on GlcN are in agreement with a number of recent

Table III

mRNA levels of MMP3 and ADAMTS4 in C-28/I2 chondrocytes treated with IL-1β or CPA (5 mM GlcN, 50 μM curcumin, or 50 μM diacerein), presented as relative quantities as compared to the untreated control group. Data were normalized using normalization factors calculated from the geometric mean of GAPDH, B2M, and HPRT1 by the geNorm software. Bold faced values indicate statistical significances as compared to the control group (P < 0.05)

	Control	IL-1β	GlcN	Curcumin	Diacerein
MMP3	1.00 ± 0.29	0.92 ± 0.27	1.88 ± 0.9	**2175.5 ± 484.3**	1.79 ± 0.38
ADAMTS4	1.00 ± 0.17	1.23 ± 0.65	2.65 ± 1.32	**119.7 ± 25.4**	1.96 ± 1.33

papers. Using Northern hybridization, Dodge *et al.*[5] also found that GlcN can stimulate mRNA levels of AGC, whereas no substantial change in MMP3 levels was induced. Another study using qPCR showed that 100 μM and 1 mM GlcN maintained COL2 and stimulated AGC gene expression in human OA chondrocytes after IL-1β exposure[6]. Similar to our results, although using bovine chondrocytes, Varghese *et al.*[33] found evidence for the relation between GlcN-induced enhancement of matrix production and concomitant inhibition of chondrocyte proliferation. In addition, those authors also showed the correlation between a reduced proliferative activity upon GlcN exposure and the appearance of a chondrocyte-specific 'spherical' morphology[33].

Regarding the potential chondroprotective effects of diacerein, studies have been conducted in both human and animal cell culture systems[10,12]. Several mechanisms of action have been discussed and consequences on the production of specific matrix components have been suggested[34–36]. To our knowledge, however, the present study is the first one demonstrating the final impact of diacerein on anabolic and catabolic marker gene transcriptions in a human chondrocyte model. Comparable results were reported by Martin *et al.*[12] in a study on bovine chondrocytes using Northern blots in which 50 μM rhein, the active metabolite of diacerein, enhanced mRNA levels of AGC and COL2 and prevented the IL-1β-mediated inhibition of synthesis of those matrix components.

In summary, our data from immortalized human chondrocytes are in line with the majority of *in vitro* studies suggesting the efficiency of GlcN and diacerein to promote the chondrocyte phenotype and to antagonize IL-1β effects on ECM components. Given the reduced protein levels of ECM components in C-28/I2 cells, studies on the protein level demand for very sensitive assays including radiolabelling[37]. Since such assays have not been performed in the present study, conclusions regarding chondroprotective effects of GlcN and diacerein on ECM proteins could not be drawn. Even though recent studies have shown a direct relationship between mRNA and protein levels of chondrocyte markers[5,32,37], this lack of protein-related experiments obviously represents a limitation of the present study. Based on this report, the application of C-28/I2 cells for the evaluation of chondroprotective action can only be suggested for studies on chondrocyte markers at the mRNA level. Moreover, the prediction of GlcN or diacerein-induced *in vivo* effects based on any *in vitro* study has to be undertaken with caution and evidence for clinical efficacy has yet to be strengthened.

Interestingly, our findings regarding the viability of chondrocytes in response to curcumin are contrary to a recent study reporting that the treatment with 50 μM curcumin for up to 48 h did not affect the viability of primary human chondrocytes at the microscopic and ultra-structural level[9]. In contrast, we found that this concentration severely damaged the integrity of the C-28/I2 cell membrane and induced cell detachment after 24 h incubation. It remains to be clarified whether this reduced viability in response to 50 μM curcumin represents a special feature of C-28/I2 cells. Noteworthy, the cellular stress caused by 50 μM curcumin in C-28/I2 cells induced a shift in the gene expression of collagen types resulting in 10-fold increased COL2/COL1 ratios (from 0.03 ± 0.06 in the control samples to 0.3 ± 0.03 in the challenged samples; $P < 0.05$). Of course, this apparently beneficial effect of curcumin is placed into perspective not only by the observed cellular damage but also by the aberrant expression of catabolic genes such as MMP3 and ADAMTS4. Therefore, our study underlines the importance of interpreting qPCR results such as COL2/COL1 ratios in consideration of additional cellular parameters.

In summary, this is the first study on a direct comparison between *in vitro* effects of the frequently discussed CPA such as GlcN, curcumin, and diacerein. In addition, the impact of diacerein on anabolic and catabolic marker gene transcriptions was presented for the first time in a human chondrocyte model. Both GlcN and diacerein promoted a differentiated chondrocytic phenotype of immortalized human C-28/I2 chondrocytes by altering proliferation, morphology, and COL2/COL1 mRNA ratios. Moreover, both agents antagonized inhibitory effects of IL-1β by enhancing AGC and COL2 as well as by reducing COL1 mRNA levels. In contrast, catabolic marker genes were not affected. Compared to GlcN, diacerein was chondroprotective at much lower concentrations which might be of interest regarding the biopharmaceutical aspects of both drugs.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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References

1. Haq I, Murphy E, Dacre J. Osteoarthritis. Postgrad Med J 2003;79:377–83.
2. Martin I, Jakob M, Schaefer D, Dick W, Spagnoli G, Heberer M. Quantitative analysis of gene expression in articular cartilage from normal and osteoarthritic joints. Osteoarthritis Cartilage 2001;9:112–8.
3. Richette P, Bardin T. Structure-modifying agents for osteoarthritis: an update. Joint Bone Spine 2004;71:18–23.
4. Clegg DO, Reda DJ, Harris CL, Klein MA, O'Dell JR, Hooper MM, *et al.* Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. N Engl J Med 2006;354:795–808.
5. Dodge GR, Jimenez SA. Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes. Osteoarthritis Cartilage 2003;11:424–32.
6. Derfoul A, Miyoshi AD, Freeman DE, Tuan RS. Glucosamine promotes chondrogenic phenotype in both chondrocytes and mesenchymal stem cells and inhibits MMP-13 expression and matrix degradation. Osteoarthritis Cartilage 2007;15:646–55.
7. Schulze-Tanzil G, Mobasheri A, Sendzik J, John T, Shakibaei M. Effects of curcumin (diferuloylmethane) on nuclear factor kB signaling in interleukin-1β-stimulated chondrocytes. Ann NY Acad Sci 2004;1030:578–86.
8. Shakibaei M, Schulze-Tanzil G, John T, Mobasheri A. Curcumin protects human chondrocytes from IL-1β-induced inhibition of collagen type II and β1-integrin expression and activation of caspase-3: an immunomorphological study. Ann Anat 2005;187:487–97.
9. Shakibaei M, John T, Schulze-Tanzil G, Lehmann I, Mobasheri A. Suppression of NF-kappaB activation by curcumin leads to inhibition of expression of cyclo-oxygenase-2 and matrix metalloproteinase-9 in human articular chondrocytes: implications for the treatment of osteoarthritis. Biochem Pharmacol 2007;73:1434–45.
10. Sanchez C, Mathy-Hartert M, Deberg MA, Ficheux H, Reginster JYL, Henrotin YE. Effects of rhein on human articular chondrocytes in alginate beads. Biochem Pharmacol 2003;65:377–88.
11. Dougados M, Nguyen M, Berdah L, Mazieres B, Vignon E, Lequesne M. Evaluation of the structure modifying effects of diacerein in hip osteoarthritis. Arthritis Rheum 2001;44:2539–47.

12. Martin G, Bogdanowicz P, Domagala F, Ficheux H, Pujol JP. Rhein inhibits interleukin-1β-induced activation of MEK/ERK pathway and DNA binding of NF-kB and AP-1 in chondrocytes cultured in hypoxia: a potential mechanism for its disease-modifying effect in osteoarthritis. Inflammation 2003;27:233–46.
13. Liacini A, Sylvester J, Zafarullah M. Triptolide suppresses proinflammatory cytokine-induced matrix metalloproteinase and aggrecanase-1 gene expression in chondrocytes. Biochem Biophys Res Commun 2005;327:320–7.
14. Richardson DW, Dodge GR. Dose-dependent effects of corticosteroids on the expression of matrix-related genes in normal and cytokine-treated articular chondrocytes. Inflamm Res 2003;52:39–49.
15. Goldring MB, Birkhead JR, Suen LF, Yamin R, Mizuno S, Glowacki J, *et al*. Interleukin-1β-modulated gene expression in immortalized human chondrocytes. J Clin Invest 1994;94:2307–16.
16. Oliver BL, Cronin CG, Zhang-Benoit Y, Goldring MB, Tanzer ML. Divergent stress responses to IL-1b, nitric oxide, and tunicamycin by chondrocytes. J Cell Physiol 2005;204:45–50.
17. Ohno S, Im HJ, Knudson CB, Knudson W. Hyaluronan oligosaccharides induce matrix metalloproteinase 13 *via* transcriptional activation of NF_B and p38 MAP kinase in articular chondrocytes. J Biol Chem 2006;281:17952–60.
18. Fay J, Varoga D, Wruck CJ, Kurz B, Goldring MB, Pufe T. Reactive oxygen species induce expression of vascular endothelial growth factor in chondrocytes and human articular cartilage explants. Arthritis Res Ther 2006;8:R189.
19. Choi SI, Park SR, Heo TR. Matrix degradation inhibitory effect of *Schisandra fructus* on human articular cartilage and chondrocytes. J Ethnopharmacol 2006;106:279–84.
20. Piana C, Gull I, Gerbes S, Gerdes R, Mills C, Samitier J, *et al*. Influence of surface modification on vitality and differentiation of Caco-2 cells. Differentiation 2007;75:308–17.
21. Toegel S, Huang W, Piana C, Unger FM, Wirth M, Goldring MB, *et al*. Selection of reliable reference genes for qPCR studies on chondroprotective action. BMC Mol Biol 2007;8:13.
22. Vandesompele J, DePreter K, Pattyn F, Poppe B, VanRoy N, DePaepe A, *et al*. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:34.
23. Fan Z, Bau B, Yang H, Soeder S, Aigner T. Freshly isolated osteoarthritic chondrocytes are catabolically more active than normal chondrocytes, but less responsive to catabolic stimulation with interleukin-1β. Arthritis Rheum 2005;52:136–43.
24. Gaissmaier C, Fritz J, Krackhardt T, Flesch I, Aicher WK, Ashammakhi N. Effect of human platelet supernatant on proliferation and matrix synthesis of human articular chondrocytes in monolayer and three-dimensional alginate cultures. Biomaterials 2005;26: 1953–60.
25. Villar-Suarez V, Calles-Venal, Bravo IG, Fernandez-Alvarez JG, Fernandez-Caso M, Villar-Lacilla JM. Differential behavior between isolated and aggregated rabbit auricular chondrocytes on plastic surfaces. J Biomed Biotechnol 2004;2:86–92.
26. Takishita Y, Hiraiwa K, Nagayama M. Effect of retinoic acid on proliferation and differentiation of cultured chondrocytes in terminal differentiation. J Biochem 1990;107:592–6.
27. Thirion S, Berenbaum F. Culture and phenotyping of chondrocytes in primary culture. In: Sabatini M, Pastoureau P, De Ceuninck F, Eds. Cartilage and Osteoarthritis. Cellular and Molecular Tools. Totowa: Humana Press 2004:1–14.
28. Tallheden T, Bengtsson C, Brantsing C, Sjögren-Jansson E, Carlsson L, Peterson L, *et al*. Proliferation and differentiation potential of chondrocytes from osteoarthritic patients. Arthritis Res Ther 2005;7: R560–8.
29. Stokes DG, Liu G, Coimbra IB, Piera-Velazques S, Crowl RM, Jimenez SA. Assessment of the gene expression profile of differentiated and dedifferentiated human fetal chondrocytes by microarray analysis. Arthritis Rheum 2002;46:402–19.
30. Darling EM, Athanasiou KA. Retaining zonal chondroyte phenotype by means of novel growth environments. Tissue Eng 2005;11:395–403.
31. Chubinskaya S, Huch K, Schulze M, Otten L, Aydelotte MB, Cole AA. Gene expression by human articular chondrocytes cultured in alginate beads. J Histochem Cytochem 2001;49:1211–20.
32. Von der Mark K, Gauss V, Von der Mark H, Muller P. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature 1977;267:531–2.
33. Varghese S, Theprungsirikul P, Sahani S, Hwang N, Yarema KJ, Elisseeff JH. Glucosamine modulates chondrocyte proliferation, matrix synthesis, and gene expression. Osteoarthritis Cartilage 2007;15:59–68.
34. Pelletier JP, Mineau F, Fernandes JC, Duval N, Martel-Pelletier J. Diacerhein and rhein reduce the interleukin 1beta stimulated inducible nitric oxide synthesis level and activity while stimulating cyclooxygenase-2 synthesis in human osteoarthritic chondrocytes. J Rheumatol 1998;25:2417–24.
35. Mendes AF, Caramona MM, de Carvalho AP, Lopes MC. Diacerhein and rhein prevent interleukin-1beta-induced nuclear factor-kappaB activation by inhibiting the degradation of inhibitor kappaB-alpha. Pharmacol Toxicol 2002;91:22–8.
36. Moldovan F, Pelletier JP, Jolicoeur FC, Cloutier JM, Martel-Pelletier J. Diacerhein and rhein reduce the ICE-induced IL-1beta and IL-18 activation in human osteoarthritic cartilage. Osteoarthritis Cartilage 2000;8:186–96.
37. Kokenyeshi R, Tan L, Robbins JR, Goldring MB. Proteoglycan production by immortalized human chondrocyte cell lines cultured under conditions that promote expression of the differentiated phenotype. Arch Biochem Biophys 2000;383:79–90.