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Chondroitin sulfate modulation of matrix and inflammatory gene expression in IL-1β-stimulated chondrocytes – study in hypoxic alginate bead cultures[1]

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Summary

Objective: To determine the effect of avian chondroitin sulfate (CS) on interleukin-1β (IL-1β)-induced expression of genes related to catabolic, anabolic and inflammatory aspects in chondrocytes cultured in hypoxic alginate beads.

Design: Articular chondrocytes from bovine metacarpal joint were isolated and cultured in alginate beads, using low oxygen atmosphere (5% O_2). After 1-week exposure to CS (1, 10 and 100 μg/ml), they were treated by recIL-1β (10 ng/ml) for 24 or 48 h, in the presence of CS. RNA was extracted and used to determine, by quantitative reverse transcription-polymerase chain reaction, the steady-state levels of mRNAs encoding several genes related to anabolic, catabolic and inflammatory aspects. Glycosaminoglycan (GAG) synthesis was also assayed by ^{35}S-sulfate incorporation.

Results: CS decreased IL-1β-induced expression of matrix metalloproteases-1, -3 and -13 and aggrecanases-1 and -2. It slightly enhanced the aggrecan core protein mRNA and the GAG synthesis. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA levels were found to be reduced by CS treatment. However, no CS-induced decrease of NO was observed in IL-1β-treated chondrocytes, whereas prostaglandin E_2 production was diminished in correlation with the COX-2 mRNA amounts. Furthermore, CS was capable of counteracting IL-1β-depressed expression of transforming growth factor-β (TGF-β) receptors.

Conclusions: CS can repress expression of genes encoding proteolytic enzymes involved in cartilage degradation. It also inhibits IL-1β-induced expression of the pro-inflammatory genes iNOS and COX-2 and restores TGF-β receptors I and II (TGF-βRI and RII) mRNA levels. These data suggest that CS may exert both chondroprotective and anti-inflammatory limited effects on articular cartilage that could have long-term beneficial action on the osteoarthritic process.

Key words: Chondroitin sulfate, Interleukin-1, Chondrocytes, Gene expression.

Introduction

Chondroitin sulfate (CS) is a glycosaminoglycan (GAG), extracted and purified from various tissues, with polysaccharide chains composed of an alternated sequence of D-glucuronic acid and D-*N*-acetyl galactosamine residues, associated by β(1 → 3) and β(1 → 4) links. Depending on the source, different non-sulfated and sulfated disaccharides units are present within the polysaccharide chains[1]. CS is a major class of GAG, which is widely distributed in all tissue extracellular matrices where it forms an essential component of proteoglycans by covalent links with proteins[2]. Because of their high degree of hydration, the CS-containing proteoglycans of articular cartilage are responsible for the visco-elastic properties of the tissue. Thus, their profound alteration in the articular cartilage of patients suffering from osteoarthritis (OA) results in great functional impairment[3].

The therapeutic use of GAG and mixtures of different polysaccharides has been markedly developed in the recent years, due to a better knowledge on their structural, biological and pharmacological properties. CS is used as a chondroprotective drug[4] and is administered in the treatment of OA, mainly by oral route, with some improvement of joint functions[5–9]. However, the cellular and molecular mechanisms underlying the symptomatic effects of CS in OA treatment remain largely unknown. Some insights into these mechanisms can be deduced from *in vitro* experiments performed on articular cartilage explants or isolated chondrocytes, albeit they do not allow extrapolation to the *in vivo* situation. CS stimulates the proteoglycan synthesis of bovine and human chondrocytes[10]. Furthermore, some anti-inflammatory properties have been attributed to CS, based on its ability to inhibit human leukocyte chemotaxis and phagocytosis, and to protect plasma membrane from oxygen reactive species[11]. Recently, *in vitro* data supporting the potential chondroprotective and anti-inflammatory effects of CS have been also reported[12,13]. However, the cellular and molecular mechanisms of these actions still remain to be elucidated. In the present study, we used for the first time, hypoxic alginate bead cultures to investigate the effects of CS on bovine chondrocytes, in order to better simulate the *in vivo* living conditions of chondrocytes. The expression activity of several genes, related to catabolic and

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anabolic aspects of the extracellular matrix, as well as inflammatory process, was determined at the transcriptional level.

Methods

CULTURE AND TREATMENT OF ARTICULAR CHONDROCYTES

Most of our experiments were performed on chondrocytes derived from the knee joints of freshly slaughtered calves. However, additional experiments were also performed on chondrocytes derived from adult retired cows (more than 10 years old). Chondrocytes were enzymatically isolated by digestion with type XIV protease (4 mg/ml; Sigma–Aldrich Co., St Quentin Fallavier, France) for 1.5 h and type I collagenase (1 mg/ml; Sigma–Aldrich Co.) overnight in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc., Cergy Pontoise, France) at 37°C. Cell culture was then performed in hypoxic conditions (5% O_2), using solutions previously equilibrated at 5% O_2 by bubbling a gas mixture deprived of oxygen. The cells were then encapsulated in alginate beads at a density of 0.75×10^6 cells/ml of low viscosity alginate gel (Sigma, 1.25% in HEPES (*N*-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) 20 mM, NaCl 0.9%) as previously described[14]. The culture flasks were placed inside a special plastic chamber, in hypoxic atmosphere (5% O_2), with DMEM previously equilibrated to 5% O_2 by gas bubbling and containing 10% fetal calf serum (FCS), 50 μg/ml sodium ascorbate and antibiotics. The beads were maintained in 75-cm^2 flask (4.5×10^6 cells/flask) for RNA extraction and in six-well plates in triplicate (1.5×10^6 cells/well) for assay of GAG neosynthesis. On day 8, the beads were incubated with 10% FCS-containing medium with CS (avian CS 1, 10 or 100 μg/ml, average MW 18 kDa, less than 1.5% protein contaminant; Pierre Fabre Laboratories, France) and medium changes were done every 2–3 days, each set of wells having the same medium replacement protocol. On day 13 or 14, interleukin-1β (IL-1β) (10 ng/ml; generous gift from Dr Soichiro Sato, Shizuoka, Japan) was added until day 15.

RNA EXTRACTION AND REAL-TIME POLYMERASE CHAIN REACTION (PCR) CONDITIONS

On day 15, the beads were dissolved in Na citrate/EDTA (Ethylenediaminetraacetic acid disodium salt) solution (55 mM Na citrate, 25 mM EDTA, in 150 mM NaCl) for 15 min at 37°C and the cells were collected. Total RNA was extracted from the cellular pellet by the guanidium isothiocyanate–phenol–chloroform procedure[15]. Following extraction, 1 μg of DNase I-treated total RNA was reverse transcribed at 37°C for 1 h. The reaction was stopped by heat inactivation (90°C for 15 min), and the resulting products were then diluted (1/100) before amplification.

Oligonucleotide primers were designed from human or bovine sequences using Primer Express software (Applied Biosystems, Foster City, CA, USA), within highly conserved regions of each complementary DNA (cDNA). BLASTN Basic Logarithmic Alignment Search Tool for Nucleotides searches were conducted on primer nucleotide sequences to ensure gene specificity (Table I).

Amplifications were carried out in triplicate, using 96-well optical plates and following all instructions of the ABI Prism 7000 SDS apparatus (Applied Biosystems). Briefly, 5 μl of diluted cDNA was mixed with both forward and reverse primers and 2× SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 15 μl. Cycling parameters were as follows: one cycle at 95°C for 10 min and 40 two-segment cycles for amplification (95°C for 10 s and 60°C for 1 min), and a dissociation protocol which is defined as a hold at 95°C for 15 s, a hold at 60°C for 20 s, and a slow ramp (20 min) from 60°C to 95°C. Single peaks were confirmed in PCR to exclude non-specific amplification, using this dissociation protocol. Serial dilutions of cDNA were also amplified to establish a standard curve and to determine the corresponding threshold cycle (CT). The 18S RNA gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. The relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method or the standard curve method depending on the efficiency of the amplification of each 18S RNA and target gene[16].

ASSAY OF GAG NEOSYNTHESIS BY ^{35}S-SULFATE INCORPORATION

On day 14, ^{35}S-sulfate (3 μCi/ml; PerkinElmer Life Sciences) was added for 24 h to assay GAG neosynthesis. The medium was collected and frozen for further analysis of NO and prostaglandin E_2(PGE_2) amounts. The beads were then washed to remove free ^{35}S-sulfate and frozen

Table I
PCR primers used in this study

Gene	Sense primer from 5′ to 3′	Antisense primer from 5′ to 3′
Aggrecan	TCG AGG ACA GCG AGG CC	TCG AGG GTG TAG CGT GTA GAG A
Aggrecanase-1	CCG CTT CAT CAC TGA CTT CCT	GGA GCC TCC GGC TTG TCT
Aggrecanase-2	AGC GCT TAA TGT CTT CCA TCC T	GTG GCT GAG GTG CAT TTG G
Type II collagen	GCA TTG CCT ACC TGG ACG AA	CGT TGG AGC CCT GGATGA
COX-1	TCA GCA CCC AGC AAA TCC T	GTG ATC TGG ATG TCA GCA CG
COX-2	GCA CCAATC TGA TGT TTG CAT TC	GGT CCT CGT TCAAAA TCT GTC TTG
IL-1β	TCT CCG ACC ACC ACT ACA GCA A	GGG GAA CTG GGC AGA CTC A
iNOS	GGC CCA GGA AAT GTT CGA A	ACC TGA TGT TGC CGT TGT TG
MMP-1	GAC CAG CAA TTT CCA AGA TTA TAA CTT	CCA AGG GAA TGG CCA AA
MMP-3	TAC GGG TCT CCC CCA GTT TC	GGT TCG GGA GGC ACA GAT T
MMP-13	TTC TTC TGG CGG CTG CAT	GGA AGT TCT GGC CCAAAC G
TGF-βRI	TTA AAA GGC GCA ACC AAG AAC	GTG GTG ATG AGC CCT TCG AT
TGF-βRII	GGA GCG GAA GAC GGA GTT G	GAC ATG CCG CGT CAG GTA CT
TGF-β1	CAT CTG GAG CCT GGA TAC ACA GT	GAA GCG CCC GGG TTG T
TGF-β2	CTG TGT GCT GAG CGC TTT T	CGA GTG TGC TGC AGG TAG TCA
TGF-β3	CAA TTA CTG CTT CCG CAA CTT G	GAT CCT GTC GGA AGT CAA TGT AGA
18S	CGG CTA CCA CAT CCA AGGAA	GCT GGAATTACC GCG GCT

until analysis. Samples were subjected to papain digestion for 18 h at 65°C (25 μg/ml in 50 mM sodium phosphate buffer containing 2 mM *N*-acetyl cysteine and 2 mM EDTA adjusted to pH 6.5)[17]. Aliquots of papain digest were used for sulfate incorporation measurements, after Alcian blue precipitation[18], in a liquid scintillation counter. dsDNA quantification was also performed to normalize sulfate incorporation among cultures, using a picogreen dsDNA assay kit (Life Technologies, Inc.). The culture medium was not assayed for its GAG content since previous reports showed that this fraction was negligible in alginate bead cultures (2–4% of the total amount)[14,17].

NO ASSAY

Nitrite levels were measured in conditioned media, using the Griess reagent and sodium nitrite as a standard[19]. Briefly, 100 μl medium was incubated with 100 μl of 0.5% sulfanilamide, 0.1% *N*-1-naphthylenediamide hydrochloride and 2.5% phosphoric acid at room temperature for 5 min in 96-well plates. Absorbance was measured at 540 nm using a spectrophotometer plate reader. NO production was normalized among cultures with dsDNA quantitation.

PGE_2 ASSAY

PGE_2 released into conditioned media was quantified using a commercially available competitive enzyme immunoassay kit (R&D Systems). Absorbance was determined at 450 nm with a wavelength correction set at 540 nm. PGE_2 production was normalized among cultures with dsDNA quantitation.

STATISTICAL ANALYSIS

At least three identical experiments were performed, using chondrocytes from different samples, which were found to give the same effects, but with variable amplitudes. The data from a representative one are shown as means ± standard deviation (SD) (PCR) or ±standard error of mean (S.E.M.) (^{35}S incorporation, NO and PGE_2 assay) of three wells per point. Statistical significance was determined by Student's *t* test using Bonferroni correction for multiple group comparisons (corrected *P*-values: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Results

EFFECTS OF CS ON MATRIX METALLOPROTEINASE (MMP) AND AGGRECANASE mRNA LEVELS

Chondrocytes were treated during 7 days with increasing concentrations of CS (1, 10 and 100 μg/ml) and with IL-1β for the latest 24 or 48 h. To determine the steady-state mRNA levels of various degradative enzymes (MMP-1, -3 and -13, and aggrecanases-1 and -2), the cells were lysed and the RNA was extracted (Fig. 1). Real-time reverse transcription-PCR (RT-PCR) revealed that CS did not significantly alter the basal levels of MMP-1, -3, and -13 and aggrecanases-1 and -2 mRNAs, whatever the concentration of CS used.

As expected, IL-1β (24 and 48 h) stimulated the expression of all these enzyme genes. Pretreatment with CS (1, 10 or 100 μg/ml) decreased the up-regulation of MMP-1 mRNA (by −22% to −32%) and aggrecanase-1 mRNAs (−20% to −40%) induced by a 24-h incubation with IL-1β.

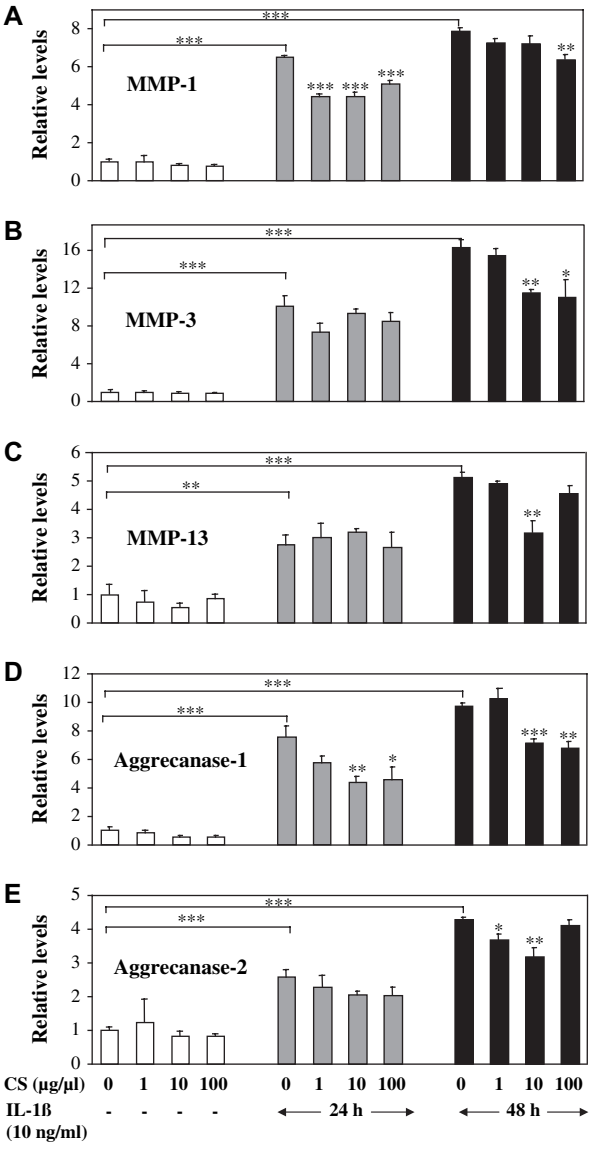

Fig. 1. Effect of CS on IL-1β-induced MMP and aggrecanase mRNA expression. Chondrocytes were treated for 7 days in the presence or absence of CS (1, 10, or 100 μg/ml). IL-1β (10 ng/ml) was added for the latest 24 or 48 h. Total RNA was extracted and used in quantitative PCR to determine steady-state mRNA levels of MMP-1 (A), MMP-3 (B), MMP-13 (C), aggrecanase-1 (D) and aggrecanase-2 (E). Data were normalized to 18S mRNA and expressed as means ± SD of triplicate samples. Statistical significance of difference between control and IL-1β treated cells, and between control, IL-1β 24 h or IL-1β 48 h and cells treated with CS was determined, using the Student's *t* test with Bonferroni correction within each group of comparisons (corrected *P*-values: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

In addition, CS was also able to reduce IL-1β-induced mRNA levels of these five degradative enzymes after 48 h, generally at the concentrations of 50 and 100 μg/ml. Thus, CS (100 μg/ml) slightly inhibited the up-regulation of MMP-1 mRNA level (−19%). CS (10 and 100 μg/ml) also reduced the increase of MMP-3, -13 and aggrecanase-1 mRNA expression and finally CS (1 and 10 μg/ml) repressed the IL-1β-induced up-regulation of aggrecanase-2 gene.

EFFECTS OF CS ON THE EXPRESSION OF IL-1β, INDUCIBLE NITRIC OXIDE SYNTHASE (INOS), AND CYCLOOXYGENASE-1/-2 (COX-1/-2)

We examined whether CS could modulate the expression of inflammatory genes implicated in joint diseases, including IL-1β, iNOS, COX-1, and COX-2 (Fig. 2). As expected, IL-1β (24- and 48-h incubation) induced a stimulatory effect on the mRNA levels of all these genes. CS had no significant effect on the basal mRNA levels of these genes. However, the IL-1β-enhanced COX-1, COX-2 and iNOS mRNA amounts were decreased by pretreatment with CS. Concentrations of 10 and 100 μg/ml CS decreased the up-regulation of iNOS and COX-2 mRNA levels induced by 24-h incubation with IL-1β (from −30% to −50% of inhibition). COX-1 and COX-2 mRNA amounts induced by a 48-h incubation with IL-1β were found to be diminished by treatment of cells with 10 or 100 μg/ml CS (−31% and −25%, respectively).

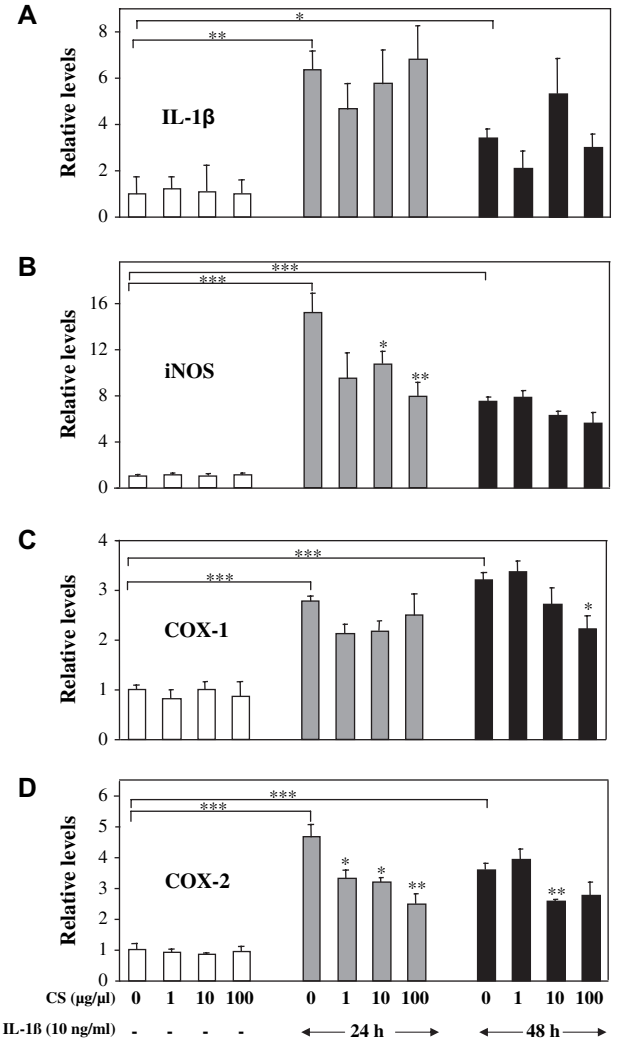

Fig. 2. Effect of CS on IL-1β-induced pro-inflammatory gene expression. Chondrocytes were treated for 7 days in the presence or absence of CS (1, 10, or 100 μg/ml). IL-1β (10 ng/ml) was added for the latest 24 or 48 h. Total RNA was extracted and used in quantitative PCR to determine steady-state mRNA levels of IL-1β (A), iNOS (B), COX-1 (C), and COX-2 (D). Data were normalized to 18S mRNA and expressed as means ± SD of triplicate samples. Statistical significance of difference between control and IL-1β treated cells, and between control, IL-1β 24 h or IL-1β 48 h and cells treated with CS was determined, using the Student's *t* test with Bonferroni correction within each group of comparisons (corrected *P*-values: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

EFFECTS OF CS ON EXPRESSION OF TYPE II COLLAGEN AND AGGRECAN CORE PROTEIN

Since IL-1β down-regulates type II collagen and aggrecan synthesis in chondrocytes[20,21], we investigated the effects of CS on the levels of type II collagen and aggrecan core protein mRNA produced in IL-1β-treated chondrocytes (Fig. 3). In agreement with the literature, we found that IL-1β decreased type II collagen and aggrecan core protein expression. CS treatment did not have significant effect on basal and IL-1β-induced levels of type II collagen mRNA. In contrast, both 10 and 100 μg/ml CS were found to slightly increase aggrecan core protein expression but the increment was only statistically significant for 100 μg/ml. These CS concentrations also exerted a repression on the IL-1β down-regulation of the gene at 24- and 48-h incubation. However, this effect was found significant for the value obtained for 100 μg/ml CS and at 24 h.

EFFECTS OF CS ON THE TRANSFORMING GROWTH FACTOR-β (TGF-β) SYSTEM

Given that TGF-β plays a crucial role in cartilage homeostasis[22–25], it was of interest to determine the effects of CS on the expression of the main TGF-β isoforms and their receptors I and II (Fig. 4). We observed that 10 μg/ml CS significantly increased the basal level of TGF-β2 mRNA

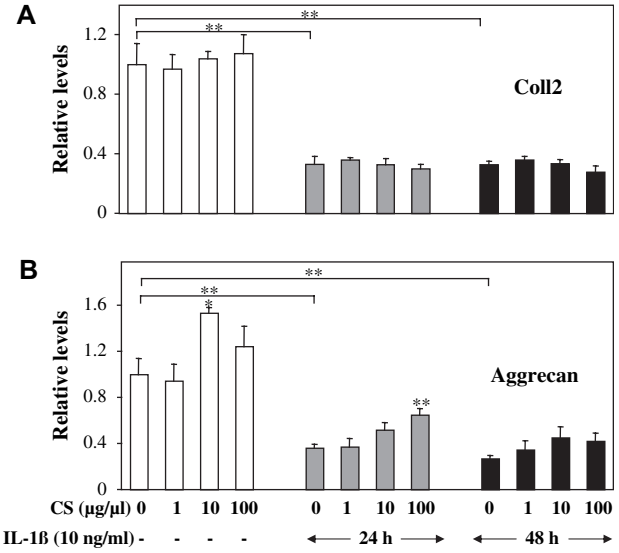

Fig. 3. Effect of CS on the expression of cartilage specific markers. Chondrocytes were treated for 7 days in the presence or absence of CS (1, 10, or 100 μg/ml). IL-1β (10 ng/ml) was added for the latest 24 or 48 h. Total RNA was extracted and used in quantitative PCR to determine steady-state mRNA levels of type II collagen (A) and aggrecan core protein (B). Data were normalized to 18S mRNA and expressed as means ± SD of triplicate samples. Statistical significance of difference between control and IL-1β treated cells, and between control, IL-1β 24 h or IL-1β 48 h and cells treated with CS was determined, using the Student's *t* test with Bonferroni correction within each group of comparisons (corrected *P*-values: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

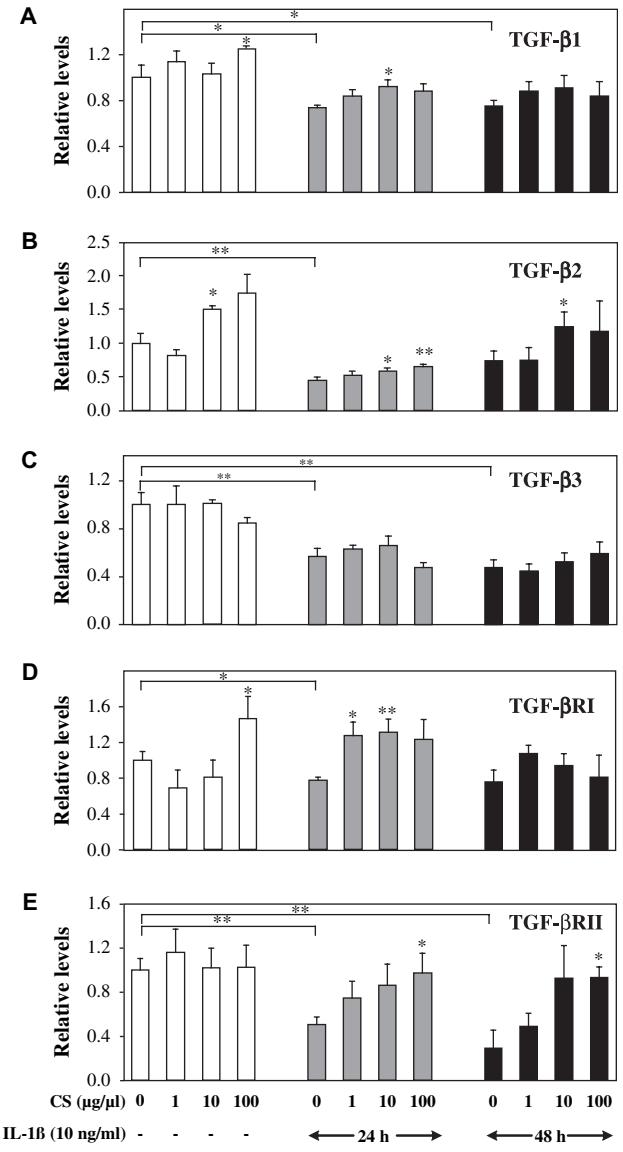

Fig. 4. Effect of CS on the expression of TGF-βs and their receptors. Chondrocytes were treated for 7 days in the presence or absence of CS (1, 10, or 100 μg/ml). IL-1β (10 ng/ml) was added for the latest 24 or 48 h. Total RNA was extracted and used in quantitative PCR to determine steady-state mRNA levels of TGF-β1 (A), TGF-β2 (B), TGF-β3 (C), TGF-βRI (D), and TGF-βRII (E). Data were normalized to 18S mRNA and expressed as means ± SD of triplicate samples. Statistical significance of difference between control and IL-1β treated cells, and between control, IL-1β 24 h or IL-1β 48 h and cells treated with CS was determined, using the Student's *t* test with Bonferroni correction within each group of comparisons (corrected *P*-values: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

(+50%) and that of TGF-βRI (+46%) at the concentration of 100 μg/ml. 24-h incubation with IL-1β decreased the expression of all genes. CS slightly reduced the cytokine down-regulation of TGF-β1 and TGF-β2 mRNA and totally prevented that of the TGF-βRI and RII genes. A 48-h incubation with IL-1β only depressed the expression of TGF-β1, TGF-β3 and TGF-βRII. CS (10 and 100 μg/ml) was capable of preventing this effect only on TGF-βRII gene, restoring the control value. However, the effect was statistically significant for 100 μg/ml only.

EFFECTS OF CS ON GAG NEOSYNTHESIS

We examined GAG neosynthesis in alginate beads using ^{35}S-sulfate incorporation[17] (Fig. 5). CS had no effect on the basal incorporation of ^{35}S-sulfate in the GAG of proteoglycans. After 24-h incubation, IL-1β induced a small diminution of ^{35}S-sulfate incorporation, whereas CS (10−100 μg/ml) slightly inhibited this reduction. CS did not significantly repressed the IL-1β inhibition of GAG synthesis after 48 h of incubation.

EFFECTS OF CS ON NO AND PGE_2 PRODUCTION

We quantified the NO and PGE_2 production into the conditioned media after CS and IL-1β treatment [Fig. 6(A)]. No significant change was observed in the CS treated cultures, whenever IL-1 was present or not. These data indicate that no correlated decrease of NO production is associated to the significant decrease of iNOS mRNA steady-state levels.

CS had no effect on the basal PGE_2 release [Fig. 6(B)]. As expected, 24- and 48-h incubations with IL-1β elevated this basal level by 5.5- and 32-fold, respectively. CS at 10 and 100 μg/ml was found to reduce this stimulation only in the 48-h incubation with IL-1β (from −24% to −45% of inhibition). However, the effect was statistically significant for 100 μg/ml CS only. Thus, in contrast to NO production, PGE_2 release was correlated to the COX-1/-2 mRNA levels, although with some delay.

EFFECTS OF CS ON CHONDROCYTES FROM AGED JOINTS

Since there may be significant differences in the response of young vs aged chondrocytes to IL-1 and CS, we performed similar experiments on cultures of cells obtained from old retired cows. We focused on the main genes which expression was found to be significantly altered by IL-1 and CS in the previous study on calf-derived cells. The concentration of 10 μg/ml CS was selected as representing a mean value, which is also close to serum levels found in patients receiving CS orally.

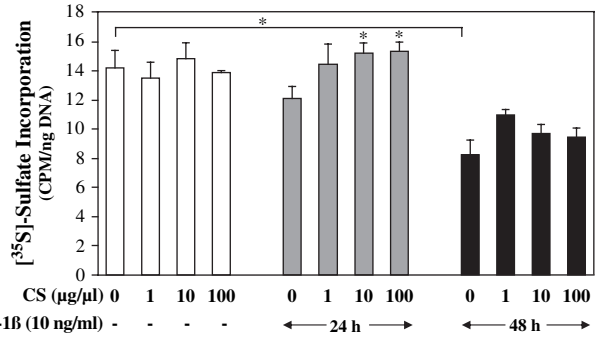

Fig. 5. Effect of CS on GAG neosynthesis. Chondrocytes were treated for 7 days in the presence or absence of CS (1, 10, or 100 μg/ml). IL-1β (10 ng/ml) was added for the latest 24 or 48 h. GAG neosynthesis, measured by ^{35}S-sulfate incorporation, was normalized to deoxyribonucleic acid content and expressed as means ± S.E.M. of triplicate samples. Statistical significance of difference between control and IL-1β treated cells, and between control, IL-1β 24 h or IL-1β 48 h and cells treated with CS was determined, using the Student's *t* test with Bonferroni correction within each group of comparisons (corrected *P*-values: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

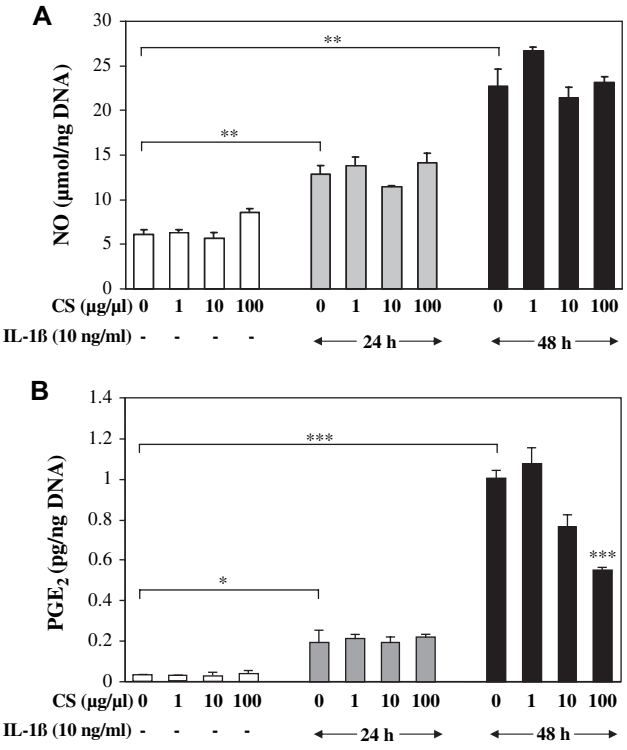

Fig. 6. Effect of CS on NO and PGE_2 synthesis. Chondrocytes were treated for 7 days in the presence or absence of CS (1, 10, or 100 µg/ml). IL-1β (10 ng/ml) was added for the latest 24 or 48 h. NO (A) and PGE_2 (B) release were normalized to deoxyribonucleic acid content and expressed as means ± S.E.M. of triplicate samples. Statistical significance of difference between control and IL-1β treated cells, and between control, IL-1β 24 h or IL-1β 48 h and cells treated with CS was determined, using the Student's *t* test with Bonferroni correction within each group of comparisons (corrected *P*-values: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$).

As can been seen in Fig. 7(A), CS treatment for 24 h induced a significant increase of aggrecan core protein mRNA (approximatively twofold), indicating that the response of this gene to CS was comparable to that found in young chondrocytes, and even greater [see Fig. 3(B)]. In the presence of IL-1β, CS was also capable to oppose the cytokine inhibition. Moreover, the stimulative effect observed was greater than in young chondrocytes [4.5-fold, compared to 0.5 in Fig. 3(B)] and even overcomes the control value (without IL-1β). Similar data were obtained for the expression of TGF-β2, the growth factor isoform which was clearly responsive to CS treatment in young cells [Fig. 7(B), compared to Fig. 4(B)]. Regarding the response of MMP-1 gene, it must be noted first that IL-1β induced a lower stimulation of the mRNA level in old vs young cells [threefold compared to eightfold increase in Fig. 1(A)]. Furthermore, this effect could be observed at 48 h rather than 24 h for calf-derived chondrocytes (24 h results for old cow chondrocytes not shown). In these conditions, CS elicited a clear inhibition of the IL-1-induced MMP-1 expression, which was greater than in the corresponding experiments with young chondrocytes [compare Fig. 1(A) to 7(A)]. Similarly, significant effect of CS on COX-1 gene occurred after 48 h. These data suggest that IL-1-responsive genes related to both matrix anabolism and catabolism, as well as pro-inflammatory genes, may be more sensitive to CS treatment in old vs young chondrocytes.

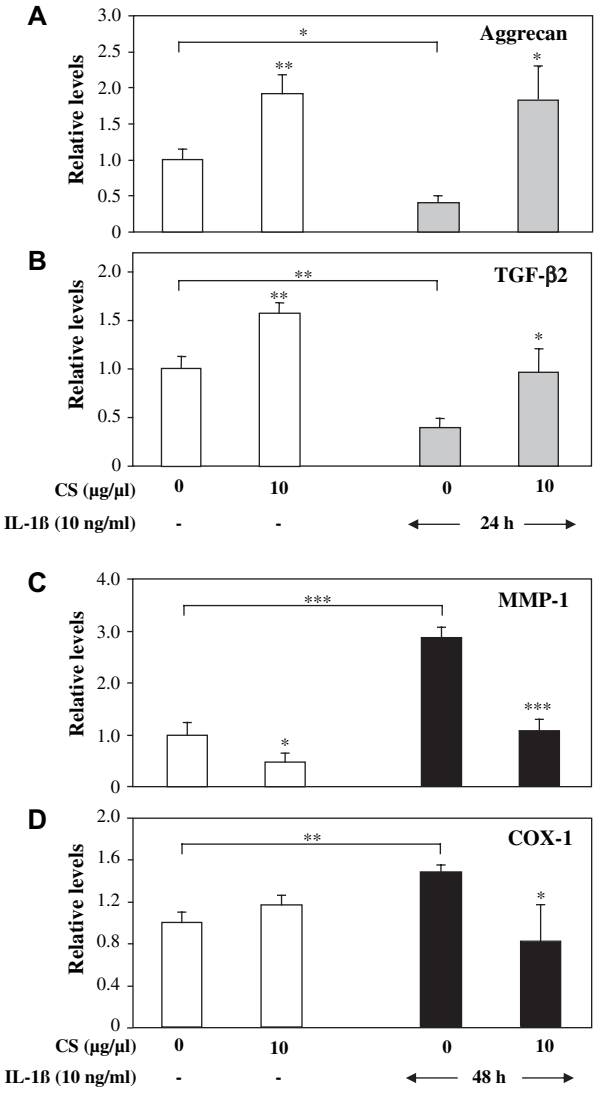

Fig. 7. Effect of CS on gene expression in chondrocytes from adult cows. Chondrocytes derived from adult cows were treated for 7 days in the presence or absence of CS (10 µg/ml). IL-1β (10 ng/ml) was added for the latest 24 or 48 h. Total RNA was extracted and used in quantitative PCR to determine steady-state mRNA levels of aggrecan (A), TGF-β2 (B), MMP-1 (C), and COX-1 (D). Data were normalized to 18S mRNA and expressed as means ± SD of triplicate samples. Statistical significance of difference between control and IL-1β treated cells, and between control, IL-1β 24 h or IL-1β 48 h and cells treated with CS was determined, using the Student's *t* test ($*P < 0.05$, $**P < 0.01$, and $***P < 0.001$).

Discussion

In the present study, we used articular chondrocytes entrapped in alginate beads and maintained in low oxygen level (5%), to better mimic the *in vivo* situation and to prevent dedifferentiation of the chondrocytes[14,26]. Chondrocytes are well adapted to cartilage low oxygen tension, which varies from 1% to 6% according to the tissue depth[27]. Therefore, the responses to IL-1β and CS in the present culture conditions are likely to reflect those of the resident chondrocytes. However, a limitation of our study was that the alginate bead procedure requires a great number of cells, so that it was difficult to also use human OA chondrocytes, which would have been more representative of the

pathology. Nevertheless, we tried to mimic the OA situation by stimulating the chondrocytes with IL-1β, as a key-cytokine of that disease. In addition, we also performed analyses on chondrocytes from old cows to determine their sensitivity to CS, as compared to that of calf-derived cells.

MMP-1 can degrade collagen, MMP-3 cleaves proteoglycans, collagens, and aggrecan link protein, MMP-13 has particular affinity for type II collagen but can also cleave aggrecan[28]. OA synovia have elevated MMP-3 mRNA[29]. IL-1β preferentially induces MMP-3 expression in human cartilage[30] and MMP-3 mRNA is elevated in the cartilage of damaged human knee and ankle[31]. MMP-13 from chondrocytes selectively enhances degradation of type II collagen in OA cartilage[32] and aggrecan at specific sites[28]. Human OA cartilage has elevated MMP-13 expression, that can be induced by IL-1β and tumor necrosis factor-α (TNF-α) in chondrocytes[33,34]. Our data show that the expression of most of the matrix-degrading enzymes produced by IL-1β-stimulated chondrocytes could be slightly, but significantly reduced by CS treatment. The transcriptional response of these genes as a function of time (24 vs 48 h) and CS concentration was found to vary. For example, MMP-1 mRNA levels were significantly decreased in a 24-h incubation with all the CS concentrations tested, whereas only the highest level of CS was found to significantly reduce this amount in the 48-h period. On the other hand, both IL-1β-induced MMP-3 and MMP-13 mRNA levels were significantly diminished after the 48-h time period only. This differential time and concentration responses of the enzymes are probably related to differences in the control mechanisms regulating the transcription machinery of their respective genes. The mechanisms whereby IL-1β induces the expression of MMPs are not fully known. However, IL-1β up-regulation of MMP-3 and MMP-13 in chondrocytes involves multiple mechanisms, including activation of ERK Extracellular regulated kinases, p38 and JNK Jun-N kinase MAPK Mitogen-activated protein kinase pathways as well as AP-1 Activating protein-1 and NF-κB Nuclear factor kappa B transcription factors[35]. Work is in progress to determine the influence of CS on IL-1β signaling pathways in chondrocytes, and to better understand these differential effects.

Our results are in agreement with previous data showing that CS down-regulated the expression of MMP-3 and MMP-13 in bovine cartilage explants[12]. This suggests that CS exerts similar effects on chondrocytes in both alginate beads and natural explant matrix. Similarly, CS (10−1000 μg/ml) was capable of inhibiting MMP-3 synthesis in IL-1β treated human OA chondrocytes[36]. More recently, the ability of CS, alone or with glucosamine, to reverse fibronectin mediated cartilage damage through reduction of MMP-3 and MMP-13 expression, has been also demonstrated[37]. All the preceding studies were performed with CS from bovine origin. Interestingly, our present findings indicate for the first time that chicken-derived CS exhibits similar properties.

Besides MMPs, a distintegrin and metalloproteinase with thrombospondin sequences aggrecanases-1 (ADAMTS4) and -2 (ADAMTS5) have been found in human OA synovial fluid and joint cartilage, together with MMP-generated aggrecan fragments[38]. Furthermore, a key role of aggrecanase-2 (ADAMTS5) in OA cartilage breakdown has been suggested by gene knock out of the enzyme, which prevented cartilage degradation in a murine model of OA[39]. In addition, it has been recently proposed that ADAMTS-mediated aggrecanolysis is preferentially destructive to cartilage function whereas MMP-mediated aggrecanolysis could be responsible for a slow turnover process of a separate pool of proteoglycans, and therefore may actually be beneficial[40]. Interestingly, we found that aggrecanase-1 expression was down-regulated by CS as early as 24 h, and that of aggrecanase-2 at 48 h, suggesting that CS could effectively reduce the transcription of these key enzymes in chondrocytes.

The steady-state level of IL-1β mRNA was not significantly modulated by CS. In contrast, the levels of IL-1β-iNOS mRNA were clearly reduced by the CS treatment, at 10 and 100 μg/ml. Nitric oxide is induced by IL-1β in chondrocytes[41] and reduces the synthesis of cartilage macromolecules[42] while it enhances their catabolism[43]. However, this effect was not accompanied by concomitant reduction in NO released in the culture medium. This finding correlates with previous data from Chan *et al.*[12,13], which reported only small decrease of NO by CS after a 24 h with IL-1β and no change after 48 h. As a possible explanation for these differences between iNOS mRNA steady-state levels and NO production, we may suggest that CS could influence the regulation of iNOS mRNA stability or/and the iNOS protein stability. Both human and murine iNOS 3′-untranslated region (3′-UTR) contain adenylate-uridylate (AU)-rich elements that are related to destabilization of mRNA of transiently expressed proteins[44,45]. AU-binding proteins either promote or prevent exonucleolytic degradation of mRNA by the exosome protein complex and thus refine the effect of (AV)-rich elements mRNA stability. In this regard, HuR Human antigen R is a ELAV (embryonic lethal abnormal vision)-like protein which binds a (AU)-rich element in the 3′-UTR of human iNOS mRNA and stabilizes iNOS[46]. After iNOS protein is expressed, it may be directed to enhanced degradation under certain conditions. For example, TGF-β inhibits IL-1β-induced NO production and iNOS expression by enhancing iNOS protein degradation in chondrocytes probably *via* proteasomes[47]. Further investigation is required to determine whether CS may influence some of these mechanisms.

In contrast, there was a correlation between COX-2 mRNA levels and PGE_2 production, after treatment with IL-1β. PGE_2 is produced in high amounts in osteo-articular diseases, and mediates inflammation, tissue destruction and inflammatory pain. COX-1 is constitutively expressed and produces low physiological levels of prostanoids, whereas the expression of the inducible isoform, COX-2, is increased in response to pro-inflammatory cytokines. COX-2 is highly expressed in RA rheumatoid arthritis and OA cartilage[48], and IL-1β enhances COX-2 expression in articular chondrocytes[49]. The present findings are in agreement with a previous study that showed CS-induced decrease of both COX-2 mRNA levels and PGE_2 production in cultured cartilage explants[13]. They can also be related to the fact that oral administration of CS in rats was found to reduce granuloma formation, as well as cell migration and lysosomal enzyme release in carrageenan pleurisy[11]. In contrast, it has been reported that the influence of CS (5−50 μg/ml) on pretranslational regulation of these selected genes was limited or lacking[50]. No clear explanation can be given for such a discrepancy. It may be due to differences in chondrocytes from various species or culture techniques (monolayer, pellet, tri-dimensional gels, and explants).

Our study showed that proteoglycan expression was slightly modulated by CS. The production of proteoglycans involves several sequential steps, from the core protein gene transcription to the enzymatic synthesis and addition of GAG chains to the protein. Each of them could potentially

be a target for CS modulation. Our findings suggested that both transcription of the core protein gene and sulfate incorporation in the neo-synthesized GAG chains are slightly up-regulated by CS treatment of chondrocytes. CS was also capable of counteracting the IL-1β-induced decrease of the core protein mRNA levels and GAG synthesis. This confirms previous study[10], and could provide a partial explanation for the chondroprotective properties of CS.

Changes in the TGF-β system of OA cartilage may greatly contribute to the irreversible process of joint degradation. TGF-βRII expression in articular cartilage was found to be dramatically depressed in the rabbit OA model[23]. A key role of that receptor in cartilage is further supported by the fact that transgenic mice expressing an inactive TGF-βRII develop joint lesions similar to human OA symptoms[24]. These data suggest that OA cartilage breakdown involves reduced responsiveness of chondrocytes to TGF-β[25]. Importantly, our results clearly showed that CS could antagonize the IL-1β down-regulation of both TGF-βRI and RII mRNA levels. This suggests that CS may contribute to maintain or restore the chondrocyte sensitivity to TGF-β and, therefore, their capacity to counteract the deleterious effects of IL-1β.

There are significant differences in the response to IL-1 of young vs aged chondrocytes. Similarly, cartilage tissue also does not display the same response to CS, depending on whether it comes from young or old animals[51]. Tissues from young animals were found rather refractory to CS-induced metabolic changes. In addition, cartilage derived from OA joints seems more responsives to polysulfated GAG than normal joints[52]. Our study also showed differences between the sensitivity of genes to CS modulation depending on the age. In adult chondrocytes, greater responses were observed for all IL-1-modulated genes. In other words, adult chondrocytes from old animals are apparently less responsive to IL-1 than cells derived from young ones, and are more sensitive to CS treatment. However, the responses were of the same type.

To evaluate the physiopathological relevance of the *in vitro* studies on CS, we may tentatively compare the concentrations used to those that may be achieved in OA patients orally treated with CS. Although the intestinal absorption of CS is still a matter of debate, orally administered tritiated CS is absorbed and found in plasma as high, low and intermediate molecular weight metabolites[11]. Furthermore, CS shows a tropism for cartilagineous tissues in rats and for knee tissues in humans, as demonstrated by scintigraphic analysis with ^{99m}Tc-labeled CS[11]. When CS was administered orally to patients as a single dose of 1200 mg/day in gastroresistant capsules, a time peak was observed at 4 h and a maximum plasma concentration of 3.8 ± 0.6 μg/ml was measured[11]. This level is within the lower part of the concentration range used in the present study (1, 10 and 100 μg/ml). However, no measurement of plasma concentrations in long-term treated patients, who generally received 1200 mg/day for at least 3 months, has already been done. Further investigation is required to determine the concentration levels, which are actually achieved in synovial fluid and cartilage treated patients.

One limitation of this study is that of steady-state levels of mRNA do not necessarily reflect protein expression and activity. However, mRNA measurements are generally good indicators for proteins that are induced rather than constitutively expressed, and simple procedures to estimate the expression and activity of several genes at the same time. Furthermore, regarding the mechanism of action of CS, our investigation did not address the possibility that a part of the exogenous IL-1β could bind to the CS added and become inactivated. A recent study with modelized CS oligosaccharides tends to support the hypothesis that two sites of interaction between IL-1β and some CS sequences might be possible[53]. Therefore, we cannot exclude that the decrease of IL-1β effects could partly result from the specific binding to CS. However, preliminary results from our laboratory suggest that CS directly activates chondrocyte signaling pathways and may interfere with those induced by IL-1β.

In conclusion, our data demonstrate that CS can decrease the expression of genes encoding proteolytic enzymes involved in cartilage degradation, including aggrecanases, whereas it slightly stimulates aggrecan synthesis. The compound also reduces the IL-1β up-regulation of COX-2 mRNA, a crucial pro-inflammatory gene in joint diseases. Furthermore, through its ability to restore the expression of TGF-βRII in chondrocytes, CS may enhance the potential of these cells to counteract IL-1β deleterious effects on cartilage. Although CS efficacy cannot be compared to that of nonsteroidal anti-inflammatory drugs, its effects on chondrocytes suggest that it can exert slight chondroprotective and anti-inflammatory actions that could have some beneficial effect on OA cartilage.

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