Variable Effects of 3 Different Chondroitin Sulfate Compounds on Human Osteoarthritic Cartilage/Chondrocytes: Relevance of Purity and Production Process

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ABSTRACT. ***Objective.*** During osteoarthritis (OA), the altered metabolism of cartilage involves proinflammatory factors and matrix metalloprotease (MMP) activity. Studies showed that chondroitin sulfate (CS) may exert a positive effect on the cartilage. Because of differences in CS in terms of purity and the production/purification process, we compared the effects of 3 different types of CS on human OA cartilage.

Methods. Three types of CS were tested: CS1 (porcine, purity 90.4%), CS2 (bovine, purity 96.2%), and CS3 (bovine, purity 99.9%). Treatment with CS at 200 and 1000 μg/ml was performed on human OA cartilage explants in the presence/absence of interleukin 1ß (IL-1ß), and the protein modulations of factors including prostaglandin E_2 (PGE_2), IL-6, and MMP-1 measured by ELISA. The CS effect on the expression of collagen type II was also investigated on OA chondrocytes using quantitative polymerase chain reaction.

Results. In the presence of IL-1ß, CS2 at 1000 μg/ml significantly inhibited IL-6 and PGE_2 production, and CS3 at 200 μg/ml markedly reduced the level of IL-6. CS1 was much less efficient at reducing the catabolic markers and in the absence of IL-1ß, it significantly increased IL-6 and MMP-1. IL-1ß significantly inhibited the gene expression level of collagen type II; only CS3 was able to limit this inhibition. CS1, in the presence or absence of IL-1ß, further markedly decreased collagen type II expression.

Conclusion. Our data indicate that among the 3 tested CS, CS1 increased production of some catabolic pathways and inhibited the gene expression level of collagen type II. Our study provides new information in the context of prescribing CS for alleviating OA symptoms, as the purity and/or production/purification of the CS compound could orient the current OA disease process toward increased catabolic pathways. (J Rheumatol First Release Feb 1 2010; doi:10.3899/jrheum.090696)

Key Indexing Terms:
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Although articular cartilage breakdown is a main characteristic of osteoarthritis (OA), its initiation is still not completely understood. However, data point to early cartilage degradation due to an increase in matrix metalloproteases (MMP) followed by an increase in proinflammatory factors[1]. Several compounds have been investigated for their positive effects on the relief of clinical symptoms and improvement of joint structural changes occurring during OA. In this context, chondroitin sulfate (CS) was shown in some studies to be a beneficial medication in the management of patients with OA[2-8]. A large-scale clinical study showed no significant symptomatic effect of CS over placebo, although the placebo response was very high[9]. Further analysis[9] revealed that the treatment effect was substantial in the subgroup of patients with moderate to severe pain. In other studies, CS was also reported to prevent the progression of structural changes in joint tissues[10-13]. CS, which belongs to the group of glycosaminoglycans (GAG), is a major component of articular cartilage matrix, and is responsible for its mechanical and elastic properties as well as other functions[14,15]. This molecule is also a major component of the extracellular matrix of other connective tis-

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sues, including bone, skin, ligaments, and tendons. CS is a sulfated GAG composed of a long unbranched polysaccharide chain with a repeating disaccharide structure of N-acetylgalactosamine and glucuronic acid[16]. Most of the N-acetylgalactosamine residues are sulfated, particularly in the 4- or 6-position (CS4 and CS6), making CS a strongly charged polyanion.

CS employed in scientific studies is generally derived from animal sources by extraction and purification processes and is mainly from bovine, porcine, chicken, and marine cartilage. However, bovine CS is the compound most often used in *in vitro* studies as well as in clinical trials. The naturally occurring CS has a molecular weight of 50 to 100 kDa and after the extraction process, its molecular weight ranges from 10 to 40 kDa, depending on the raw material.

In articular tissue, CS was shown to reduce some proinflammatory factors and proteases, to modify the cellular death process, and to improve the anabolic/catabolic balance of extracellular cartilage matrix[17-27]. CS alone or in combination with glucosamine also promotes synovial hyaluronic acid production, suggesting a beneficial effect of maintaining viscosity properties in the joint[28,29]. Finally, CS in combination with glucosamine was also reported to decrease the proresorptive properties of human OA subchondral bone[30]. It should be noted that results of scientific studies and clinical trials are directly related to the quality of material used, as analyses of different products revealed that the species or tissue of origin could result in great differences in CS structural organization or disaccharide composition[31-33]. Because of differences in molecular composition, which is dependent upon the species, tissue of origin, purity, and production/purification process, we investigated the effects of 3 types of CS on OA cartilage. Our data reveal information on the effects of CS from different species and with different percentages of purity on the catabolic and anabolic factors in cartilage. Data showed that different CS compounds should be taken with care, as the purity of the compound could orient the OA disease process toward either decreased or increased catabolic pathways.

MATERIALS AND METHODS

Specimen selection. Human OA specimens were obtained from the femoral condyles and tibial plateaus of patients undergoing total knee arthroplasty (10 women/2 men; mean age ± SD: 65 ± 11 yrs). All patients were evaluated as having OA according to the American College of Rheumatology clinical criteria[34]. At the time of surgery, the patients had symptomatic disease requiring medical treatment in the form of acetaminophen, nonsteroidal antiinflammatory drugs, or selective cyclooxygenase-2 inhibitors. None had received intraarticular steroid injections within 3 months prior to surgery. The Institutional Ethics Committee Board of the Notre-Dame Hospital approved the use of the human articular tissues and patients gave informed consent.

Cartilage explant culture. Human OA cartilage explants (~150 mg) were incubated in Dulbecco's modified Eagle's medium (DMEM; Wisent, Saint-Bruno, QC, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS; PAA Laboratories, Etobicoke, ON, Canada) and an antibiotics mixture (100 units/ml penicillin base and 100 μg/ml streptomycin base; Wisent) at 37˚C in a humidified atmosphere of 5% CO_2/95% air. Cartilage explants were treated for 7 days (for protein determination) in DMEM containing 10% FBS in the presence or absence of interleukin 1ß (IL-1ß; 1 ng/ml; R&D Systems Inc., Minneapolis, MN, USA) without/with 200 or 1000 μg/ml CS. The medium was replaced once (after 3 days) and at the end of the incubation period, a conditioned medium was mixed. Protein determinations thus reflect the accumulation of 7 days.

The characteristics of each CS used in this study are shown in Table 1. The origin of the CS was confirmed by polymerase chain reaction (PCR), through the determination of DNA fragments (data not shown). CS content and molecular weight (average molecular weight) were respectively determined by cetylpyridinium chloride (CPC) titration with a coefficient of variation about 0.4%[35,36] and gel permeation chromatography with refractive index detection. It is known that the CPC titration method could also measure other polyanions; however, when other sulfate-containing compounds are not present, this method provides a reliable measure. We then looked at the presence of other natural polysaccharides in the CS products by electrophoresis separation of GAG species on cellulose acetate[36]. Results showed the absence of other GAG in the CS preparation (data not shown). The protein content was determined by the Lowry method. The intrinsic viscosity was determined according to the European Pharmacopeia[35]. Chloride, sulfate, and oxalate content were assessed by ion chromatography (IC) with chemical suppression and conductivity detection, and sodium by IC with conductivity detection. The disaccharide composition was determined by enzymatic hydrolysis with Chondroitinase ABC, followed by IC with UV detection (method adapted from Korean Food and Drug Administration).

All 3 CS, originating from different manufacturers, were provided by Bioibérica S.A. (Barcelona, Spain).

Chondrocyte culture and treatment. Chondrocytes were released from full-thickness strips of cartilage followed by sequential enzymatic digestion at 37˚C, as described[37]. Cells were seeded at high density (10^5 cells/cm^2) in tissue culture flasks, and cultured to confluence in DMEM supplemented with 10% FBS and an antibiotics mixture at 37ºC in a humidified atmosphere. To ensure phenotype, only first-passage cultured chondrocytes were used.

At confluence, monolayer chondrocytes were treated for 20 h [for messenger RNA (mRNA) determination] in DMEM containing 0.5% FBS in the presence or absence of IL-1ß (100 pg/ml) with/without 200 μg/ml or 1000 μg/ml of CS1, CS2, or CS3. Preliminary experiments were performed for the time-curve effect of the 3 different CS and treatment revealed a maximum effect at 20 h.

Table 1. Chondroitin sulfate (CS) characteristics.

Characteristic	CS1	CS2	CS3
Species	Porcine	Bovine	Bovine
CS content, %	90.4	96.2	99.9
Molecular weight*, kDa	12.9	13.8	15.12
Protein, %	7.4	3.3	ND
Intrinsic viscosity, m^3/kg	0.034	0.036	0.040
Chlorides, %	0.70	0.02	0.34
Free sulfates, %	0.75	0.05	0.14
Oxalate, %	0.021	ND	0.01
Sodium, %	7.10	6.75	7.05
ΔDisaccharide 0–S, %	5.9	5.1	5.7
ΔDisaccharide 4–S, %	78.3	72.7	62.8
ΔDisaccharide 6-S, %	15.8	21.3	31.5
ΔDisaccharide 2,6-S, %	ND	0.4	ND
ΔDisaccharide 4,6-S, %	ND	0.5	ND

* Indicates average molecular weight. ND: below the detection limit.

RNA extraction, reverse transcription (RT), and real-time PCR. Total cellular RNA from human OA chondrocytes was extracted with the TRIzol™ reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's specifications and treated with the DNA-free DNase treatment and removal kit (Ambion, Austin, TX, USA) to ensure complete removal of chromosomal DNA. The RNA was quantitated using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR, USA). The RT reactions were primed with random hexamers and real-time quantitation of mRNA was performed as described[30] in the Rotor-Gene RG-3000A (Corbett Research, Mortlake, Australia) with the 2× Quantitect SYBR Green PCR Master Mix (Qiagen, Mississauga, ON, Canada), used according to the manufacturer's specifications. In brief, 45 ng of the cDNA obtained from the RT reactions were amplified in a total volume of 50 μl consisting of 1× Master mix, uracil-N-glycosylase (Epicentre Technologies, Madison, WI, USA) 0.5 U, and the gene-specific primers, which were added at a final concentration of 200 nM. The primer sequences were 5'-AGT TTC AGG TCT CTG CAG GT (sense), 5'-AAC TGG CAA GCA AGG AGA CA (antisense; collagen type II α1) and 5'-CAG AAC ATC ATC CCT GCC TCT (sense) and 5' GCT TGA CAA AGT GGT CGT TGAG [antisense; glyceraldehyde phosphate dehydrogenase (GAPDH)]. The primer efficiency for the test gene was the same as for the GAPDH gene. The standard curve was generated with the same plasmid as the target sequence. The data were collected and processed with Corbett analysis software and given as corresponding to the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected. The C_t was then converted to number of molecules, and the values for each sample were calculated as the ratio of the number of molecules of the target gene/number of molecules of GAPDH. Data are expressed as arbitrary unit over control, which was assigned a value of 1.

Other determinations. The levels of prostaglandin E_2 (PGE_2; Cayman Chemical Company, Ann Arbor, MI, USA), IL-6 (R&D Systems), and MMP-1 (R&D Systems) were quantified with the Fluorokine MAP Human kit using the LiquiChip Luminex apparatus (Qiagen), according to the manufacturer's specifications. All determinations were performed in duplicate for each cell culture. Data are expressed as the mean ± SEM. Statistical significance was assessed by the Wilcoxon matched pairs test.

RESULTS

PGE_2 production in the absence of IL-1ß was very low (Figure 1A, n = 6). Data showed that none of the CS tested had a statistically significant effect. In the presence of IL-1ß (Figure 1B, n = 6), production of this factor was significantly increased by about 16-fold ($p < 0.03$). CS2 at 1000 μg/ml markedly and significantly ($p < 0.03$) inhibited PGE_2 production.

The basal level of IL-6 was, as for PGE_2, very low and IL-1ß increased its level by about 34-fold ($p < 0.03$; Figure 2, n = 6). In the absence of IL-1ß (Figure 2A), CS1 markedly increased the basal IL-6 level in a concentration-dependent fashion, and statistical significance was reached for the highest concentration tested. None of the other CS had any statistically significant effect. In the presence of IL-1ß (Figure 2B), each CS at both concentrations inhibited the IL-1ß-induced IL-6, with a statistically significant effect reached for CS2 at 1000 μg/ml ($p < 0.03$). CS3 at 200 μg/ml showed a similar decrease to that of CS2 at 1000 μg/ml; however, this did not quite reach statistical significance ($p < 0.06$).

The levels of MMP-1 (Figure 3, n = 6) in the absence of IL-1ß (Figure 3A) were markedly induced by CS1 with a statistically significant difference ($p < 0.03$) found at 1000 μg/ml. As expected, IL-1ß induced a statistically significant increase in MMP-1 production levels ($p < 0.03$; Figure 3B). In the absence or presence of IL-1ß, none of the other CS had any true effect.

The expression level of collagen type II α1 was significantly ($p < 0.03$) decreased by CS1 at both concentrations in the absence of IL-1ß (Figure 4A, n = 6). In the presence of IL-1ß (Figure 4B, n = 6), the collagen type II α1 expression level was significantly decreased ($p < 0.03$). CS1 at both concentrations further decreased the level of collagen type II α1 with a trend toward statistical significance ($p < 0.06$), whereas CS3 limited this inhibition and CS2 was without effect.

DISCUSSION

In the past few decades, research has aimed to understand the exact pathophysiological pathways of OA in order to develop specific therapies that could alleviate its progression. Several candidates have been tested, and CS has been investigated as a potential therapeutic agent for the treatment of OA[3,4,6]. Because CS compounds different in terms of purity and purification are currently being commercialized and the literature revealed that disparity in effects could occur depending on the CS structural organization[31], we compared the effects of 3 different CS compounds on human OA cartilage.

One could question the relevance of the high CS concentrations generally employed in *in vitro* studies. It is known that CS is absorbed after oral administration and a high content of labeled CS is found in the synovial fluid and cartilage[38-40]. However, is the concentration generally used in *in vitro* studies, about 200 μg/ml, attainable in articular tissues *in vivo*? As treatment with CS is characterized by a slow onset of action, with a maximal clinical effect being attained after several months (3 to 6 months), it appears necessary to use a high concentration for *in vitro* studies in order to reproduce an effect observed *in vivo* after months of treatment.

In general, our study showed that CS1 increased the production of some catabolic factors and decreased the gene expression level of an anabolic factor; this occurred mostly in the absence of IL-1ß. In contrast, CS2 at a concentration of 1000 μg/ml and CS3 at 200 μg/ml decreased the production of some catabolic factors; this occurred in the presence of IL-1ß. On collagen type II α1, only CS3 reduced the inhibitory effect of IL-1ß.

MMP-1 was chosen for our study, as this enzyme has been closely linked to the degradative process that occurs in the cartilage because of its activity not only on collagen but also on a broad range of noncollagenous extracellular macromolecules. Surprisingly, data from our study show that upon CS treatment in the absence of IL-1ß, CS1 significantly ($p < 0.03$) increased the MMP-1 and IL-6 levels. CS2 and CS3 had no effect in the absence of IL-1ß, but in

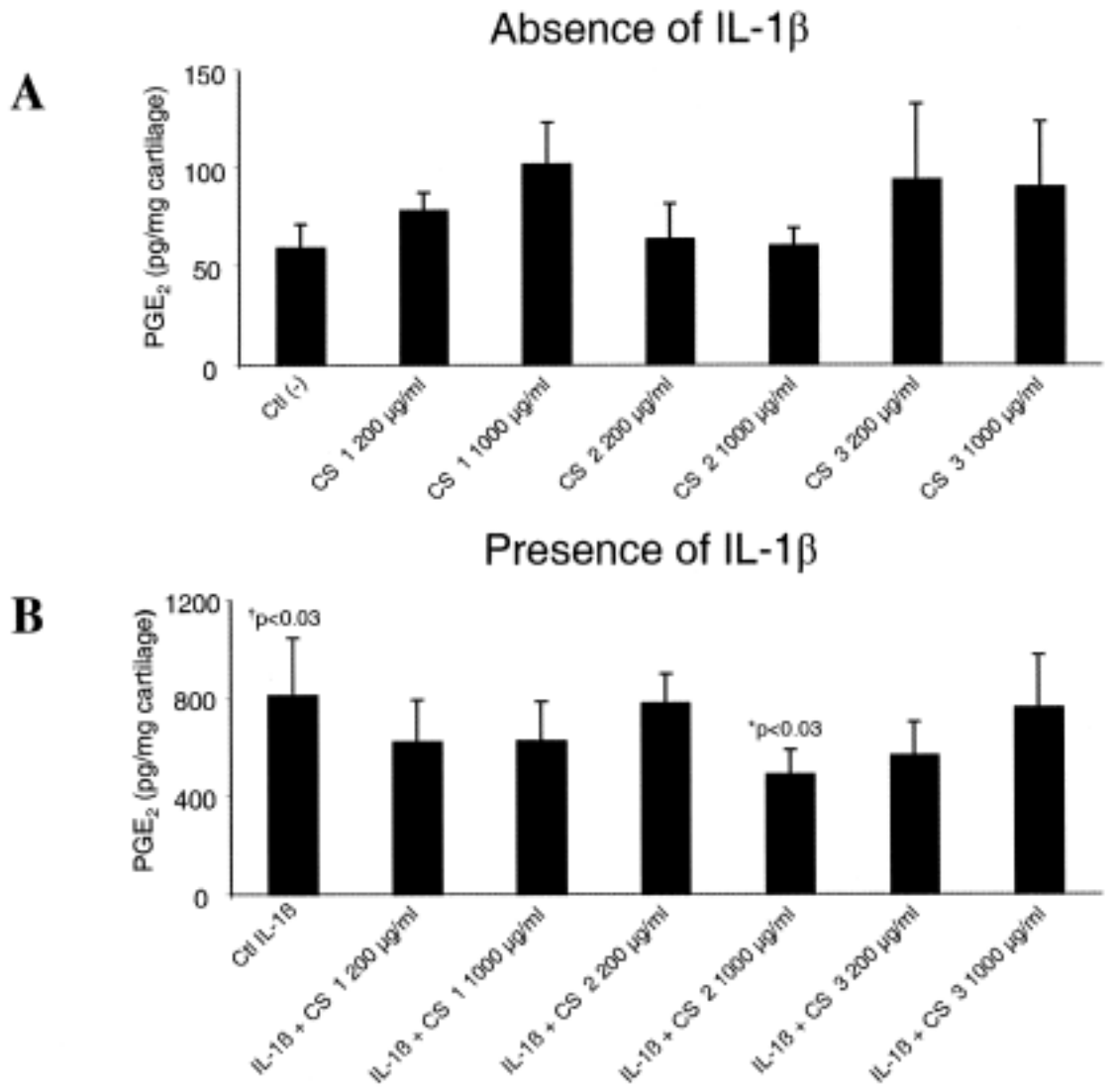

Figure 1. Effect of chondroitin sulfate (CS) 1, CS2, and CS3 on modulation of PGE_2 level (pg/mg cartilage) in human OA cartilage explants in the absence (A) and presence (B) of IL-1ß. Data are from 6 independent experiments and expressed as mean ± SEM. *p value (Wilcoxon matched-pairs test) indicates the difference between control (Ctl) and CS in the presence of IL-1ß; and †between Ctl and Ctl + IL-1ß-treated specimens.

its presence, CS2 at 1000 μg/ml and to a lesser extent CS3 at 200 μg/ml were the most efficient at reducing production of PGE_2 and IL-6, 2 catabolic factors involved in cartilage matrix degradation, the levels of which have been found to be significantly elevated in OA tissues[41-45]. CS1 significantly inhibited the anabolic macromolecule collagen type II in the absence of IL-1ß, and a trend toward a reduction was found in the presence of this cytokine.

The effect of CS2 and CS3 on IL-6 is interesting as this cytokine is also involved in degradation/catabolic effects in 2 other articular tissues, the subchondral bone and the synovial membrane. In human OA subchondral bone osteoblasts, IL-6 was demonstrated to increase the level of membranous receptor activator of nuclear factor-κB ligand (RANKL; an osteoclastogenesis factor) in favor of bone resorption[46]. Similarly, CS was shown to inhibit the RANKL expression level on these cells[30]. Yet, by inhibiting IL-6 production in cartilage, CS could also indirectly affect the subchondral bone remodeling process, as there is substantiated evidence for cross-talk between cartilage and subchondral bone[47]. Synovial membrane inflammation also plays an important role in the progression of joint tissue lesions, and among the proinflammatory cytokines, IL-6 was found to be significantly increased in human OA synovial fluid[42]. Although not tested, it would be of interest to investigate the level of this factor in the synovial fluid *in vivo* upon CS treatment and its correlation with IL-1ß, as data from the GAIT clinical trial[9] demonstrated that treatment with CS was associated with a statistically significant decrease in the incidence of joint swelling, effusion, or both.

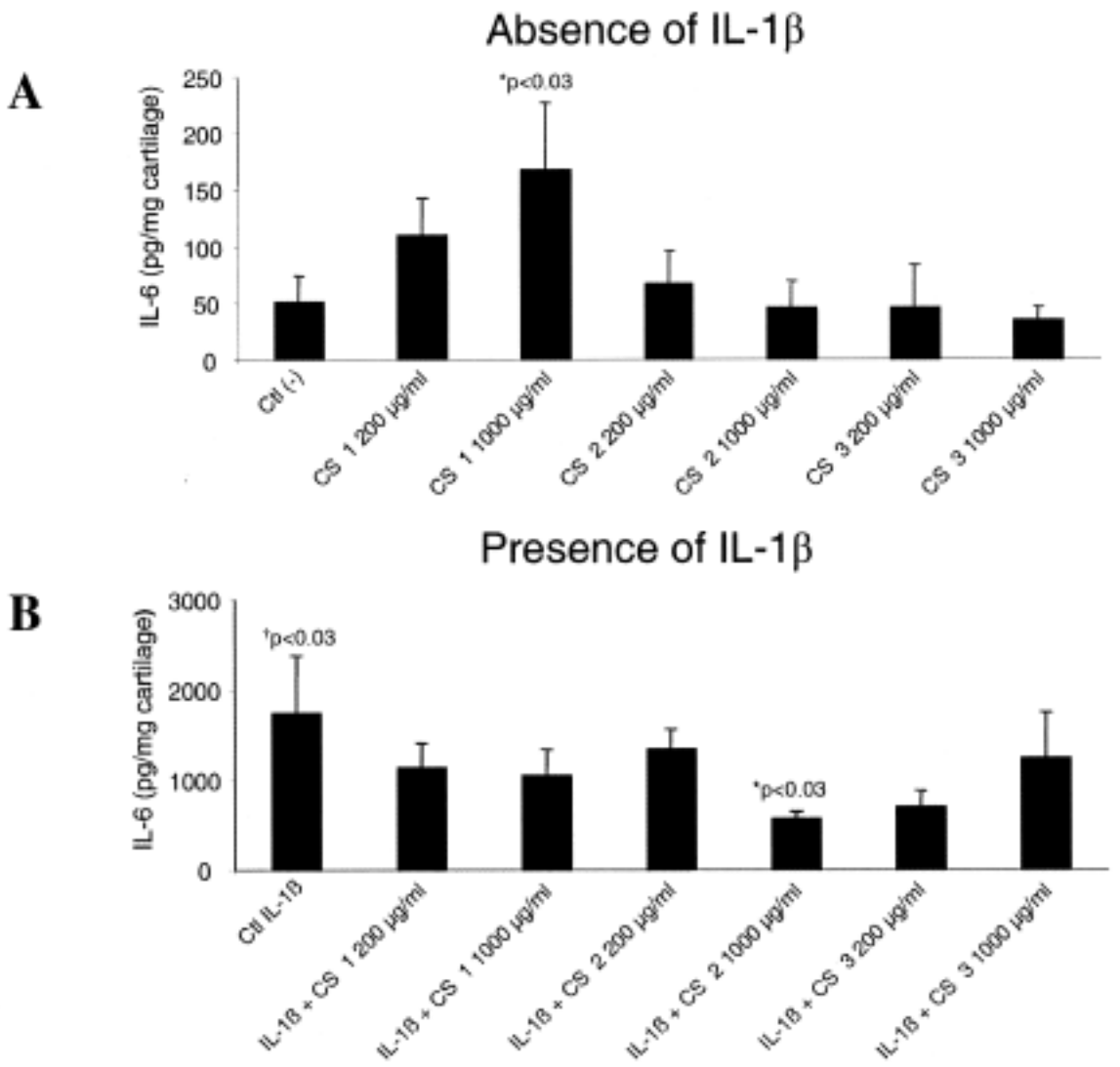

Figure 2. Effect of chondroitin sulfate (CS) 1, CS2, and CS3 on modulation of IL-6 level (pg/mg cartilage) in human OA cartilage explants in the absence (A) and presence (B) of IL-1ß. Data are from 6 independent experiments and expressed as mean ± SEM. *p value (Wilcoxon matched-pairs test) indicates the difference between control (Ctl) and CS in the presence or absence of IL-1ß; and †between Ctl and Ctl + IL-1ß-treated specimens.

It could then be that the reduction in joint swelling and effusion was due to a reduction in proinflammatory cytokines, including IL-6. This would concur with an *in vivo* study on an animal model (murine collagen-induced arthritis) in which the serum level of IL-6 was diminished following CS treatment[48].

The differences in purity and properties of the CS could be responsible for the disparity in effects. Further, different bioavailability and pharmacokinetic variables have also been reported to change depending on the CS structural characteristics and origin[31,32]. The position and percentage of sulfate groups are generally in relation to specific animal sources. Although CS are mainly composed of disaccharides sulfated at carbon 4 or 6 (6-S, 4-S) positions, various percentages are found depending on the origin. Thus, as the sulfation properties vary and are key components for CS efficacy/activity and specialized functions, their origin is also of major significance[49,50]. For example, for another GAG, the dermatan, the 6-*O* sulfation was found to be much more efficient at exerting its anticoagulant activity compared to 4-*O* sulfation[51]. More specifically for chondroitin, CS 4-S was found more effective than the 6-S as an antioxidant[52,53], but the loss of chondroitin 6-*O*-sulfotransferase 1, thus an inhibition in 6 sulfation, results in severe human chondrodysplasia and is of importance in chondrocyte differentiation[54]. Although we made no attempt to correlate the disaccharide composition with the effect of the CS, it is notable that among the 3 CS tested, there were some differences. While

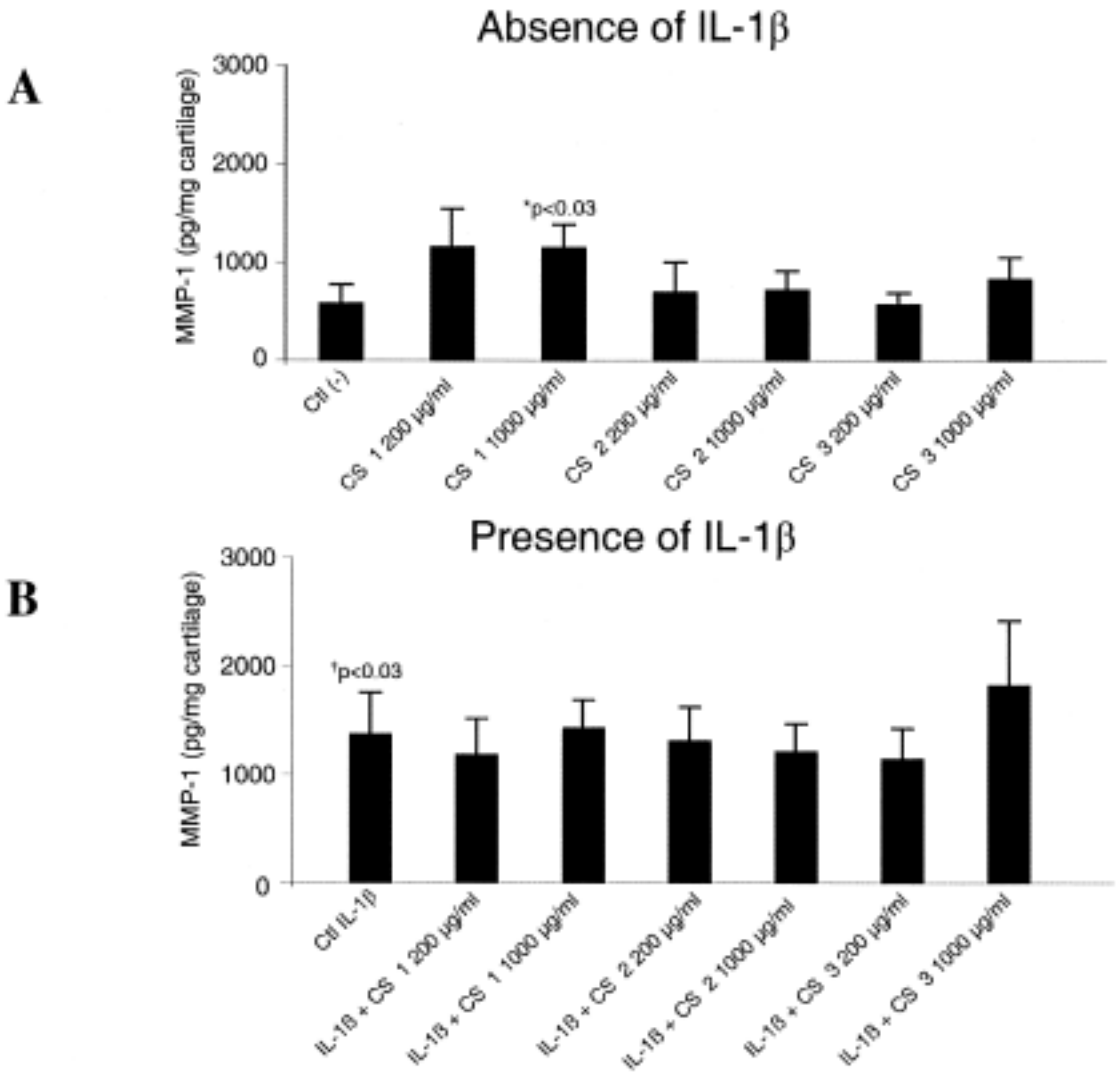

Figure 3. Effect of chondroitin sulfate (CS) 1, CS2, and CS3 on modulation of MMP-1 level (pg/mg cartilage) in human OA cartilage explants in the absence (A) and presence (B) of IL-1ß. Data are from 6 independent experiments and expressed as mean ± SEM. *p value (Wilcoxon matched-pairs test) indicates the difference between control (Ctl) and CS in the presence or absence of IL-1ß; and †between Ctl and Ctl + IL-1ß-treated specimens.

the percentage of nonsulfated disaccharides was similar, the porcine CS, CS1, had the lowest amount of 6-S disaccharide, about 25% and 50% less than CS2 and CS3, respectively, and the highest level of 4-S disaccharide by about 7% and 24%, respectively. Thus, even if further studies are required to establish a correlation between chemical structure and activity in these cells, these differences might be an explanation for the different effects among these CS.

However, a major difference between the 3 tested CS could also be their final purity. In this study the CPC titration coefficient of variation was very low (around 0.4%), which suggests that the differences between the 3 CS products (differences of 4%–10%) are not attributed to the analytical procedure but to differences in the impurity profile and the integrity of the molecules. Thus the extraction process and purity of the CS raw material are key points in the preparation if produced for pharmaceutical and nutraceutical applications. CS molecules should be obtained through a selective and solid extraction process, which includes enrichment and purification based on the chemical structure and molecular weight, and solvent fractioning steps. In contrast to other extraction procedures that involve potent and nonspecific oxidation in alkaline conditions, the selective and solid process does not modify the original chemical and structural properties, thereby producing substances with high molecular weight and purity (such as CS3). Data showing that CS1 had the highest content of free sulfates (0.75%) combined with its lower molecular weight (compared to the 2 other CS) suggest a higher desulfation and a higher depolymerization during its manufacturing

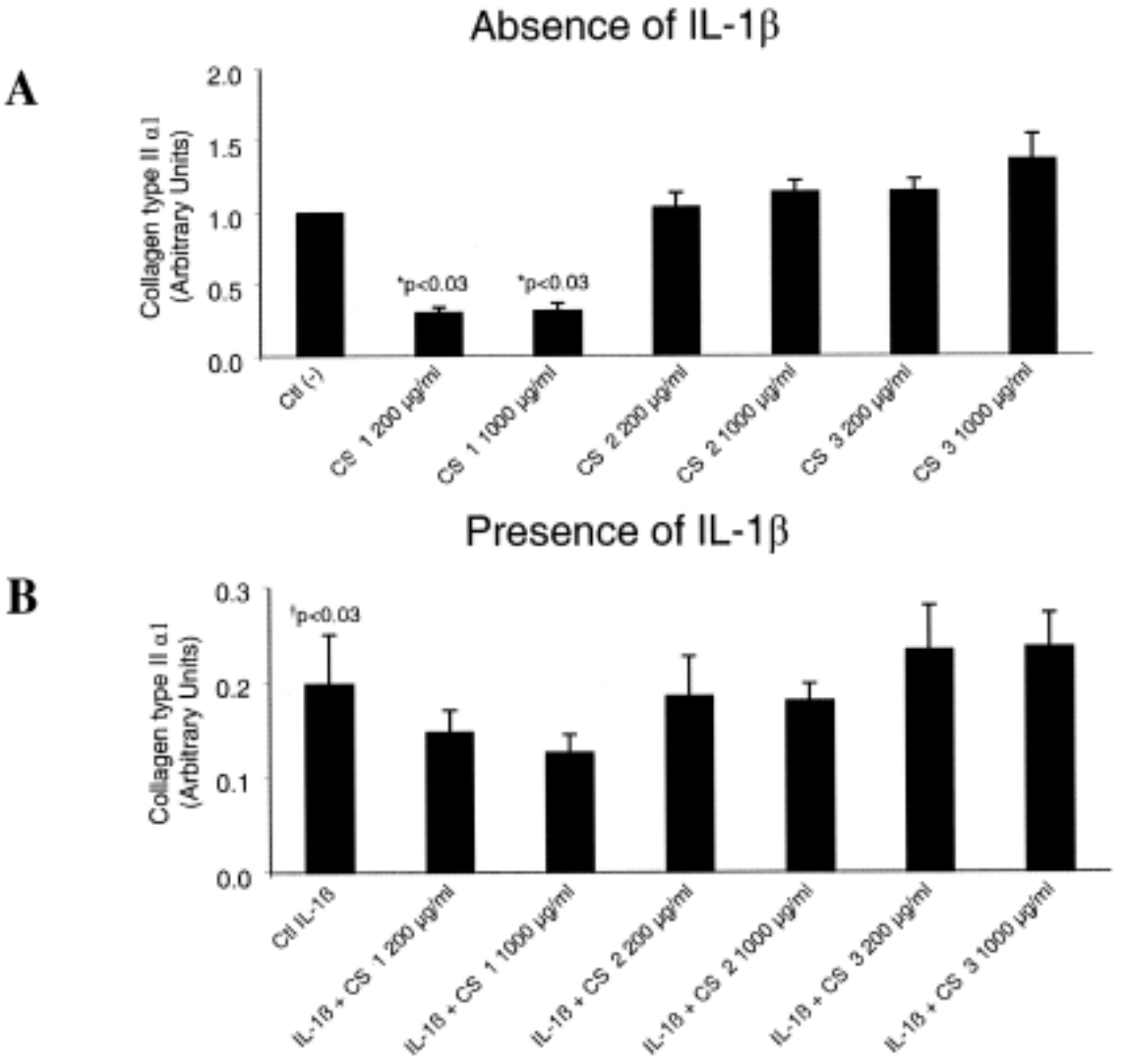

Figure 4. Effect of chondroitin sulfate (CS) 1, CS2, and CS3 on the gene expression level of collagen type II α1, arbitrary units in which the control without IL-1ß [Ctl (–)] was given a value of 1, in human OA primary chondrocytes cultured in the absence (A) and presence (B) of IL-1ß. Data are from 6 independent experiments and expressed as mean ± SEM. *p value (Wilcoxon matched-pairs test) indicates the difference between Ctl and CS in the presence or absence of IL-1ß; and †between Ctl and Ctl + IL-1ß-treated specimens.

process. Therefore, the purification protocols are of major importance. Indeed, the purification conditions produce extracts with variable levels of CS and hence inconsistent grades of purity explained by the presence of side products.

The nature of these products could contribute to the overall biological and pharmacological actions of these agents and also be involved in the differential effect between the CS[55]. It is known that other natural polysaccharides could be present in the CS preparation; however, data showed the absence of such products in the CS tested. Although many other contaminants could explain the increase in some catabolic pathways by CS1, the highest protein levels found in CS1 compared to the other CS could have been responsible for such events.

Hence, when used as a therapeutic, the percentage of purity of commercial CS should be well documented and investigated for any unspecific effect. The lower level of purity of CS1 could explain the unspecific mechanism in the OA chondrocytes in favor of catabolic action, while the level of purity of CS2 might explain why a higher concentration, 1000 μg/ml, was necessary to obtain an effect similar to that of CS3 at 200 μg/ml.

Our study provides new information on the effects of different CS compounds on human OA cartilage/chondrocyte metabolism. Our data indicate that among the 3 CS compounds tested, CS1 induces increased catabolic pathways, an action that could be caused by its properties and purity.

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