Anti-arthritic Action Mechanisms of Natural Chondroitin Sulfate in Human Articular Chondrocytes and Synovial Fibroblasts

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To clarify the exact anti-arthritic action mechanisms of chondroitin sulfate (CS), we evaluated the effects of CS derived from shark cartilage (CS-SC) composed mainly of chondroitin-6-sulfate and porcine trachea cartilage (CS-PC) composed mostly of chondroitin-4-sulfate on the functions of human articular chondrocytes and synovial fibroblasts. Both CS-SC and CS-PC (from 1 to 100 μg/ml) effectively suppressed the interleukin (IL)-1β (10 ng/ml)-enhanced gene expression of aggrecanase-1/a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS)-4 and aggrecanase-2/ADAMTS-5 in articular chondrocytes embedded in alginate beads and synovial fibroblasts. In addition, CS-SC and CS-PC overcame the IL-1β-mediated suppression of the aggrecan core protein mRNA, and suppressed the IL-1β-enhanced collagenase-3/matrix metalloproteinase (MMP)-13 gene expression in chondrocytes. CS-PC, but not CS-SC effectively recovered the IL-1β-reduced gene expression of tissue inhibitor of metalloproteinases (TIMP)-3 in chondrocytes, and enhanced the production of TIMP-1 in synovial fibroblasts. It is noteworthy that CS is able to modulate the function of synovial fibroblasts as well as that of chondrocytes. Therefore, CS is very likely to be multifunctional chondroprotective material for degenerative arthritic diseases.

Key words chondroitin sulfate; tissue inhibitor of metalloproteinase; aggrecanase; cartilage; chondrocyte; synovial fibroblast

Osteoarthritis (OA) is the most common form of arthritis, and is characterized by cartilage destruction with loss of compressive and tensile strength properties. Cartilage is a specialized connective tissue that consists mainly of extracellular matrix (ECM) including type II collagen, hyaluronan, and aggrecan, and a small amount of chondrocytes. Aggrecan, a large aggregating proteoglycan, interacts with hyaluronan and link protein to form large aggregates that are immobilized in the cartilage by type II collagen, resulting in a dense network of collagen fibrils and drawing of water into cartilage. These ECM components are enzymatically degraded in OA. The loss of aggrecan is considered to be a critical early event of OA that is followed by the degradation of collagen fibrils.

Aggrecanases that specifically cleave the Glu-374-Ala bond of aggrecan core protein are thought to be the most critical enzymes for the cartilage ECM degradation under physiological and pathological conditions. The products of this cleavage have been found in synovial fluid of patients with OA, suggesting that aggrecanase exclusively participates in the catabolism of cartilage matrices in OA. Among the several aggrecanases, aggrecanase-1/a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS)-4 and aggrecanase-2/ADAMTS-5 have been characterized as the critical enzymes for pathological cartilage destruction. In particular, ADAMTS-5 has been shown to play crucial roles in cartilage destruction in experimental osteoarthritis. Therefore, aggrecanases are the most important molecular targets for the treatment of OA. On the other hand, matrix metalloproteinases (MMPs), especially collagenase-3/MMP-13 that prefers to degrade type II collagen, have also been shown to play a significant role in cartilage destruction. Active MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs)-1, -2, and -3. Thus, the enzymic activity of MMPs is critically regulated by a quantitative balance between MMPs and TIMPs. Besides, TIMP-3 is also known as a potent inhibitor of ADAMTS-4 and -5, and thus it closely participates in the control of aggrecanase activity.

The various medicines are applied for the treatment of rheumatoid arthritis (RA), currently available medical therapies primarily address only the treatment of joint pain in OA including non-steroidal anti-inflammatory drugs (NSAIDs). Natural chondroitin sulfates are widely used for the treatment of joint pain in the world. Chondroitin sulfate (CS) is a glycosaminoglycan with polysaccharide chains composed of an alternate sequence of D-glucuronic acid and D-N-acetyl galactosamine linked by β(1→3) and β(1→4) bonds, and it is widely distributed in various connective tissues; it is particularly abundant in cartilage, skin, corpus vitreum, and blood vessels. Most CS exists as the sugar chains of aggrecan in the cartilage, and its high water retaining capacity ensures proper cartilage hydration. Thus, CS contributes to the visco-elastic property of the cartilage, and a reduced level of CS in the cartilage has been reported to be a risk factor for arthritic diseases in elderly people. CS is well recognized as a symptomatic slow-acting drug for OA (SySADOA), the same as diacerein and avocado unsaponifiables, in a few European countries. In addition, CS is popular as a dietary supplement expected to alleviate joint pain in North America. In Japan, CS is available as an over-the-counter drug for knee joint pain. However, the therapeutic action mechanisms of CS remain unclear.

In the present study, we provide novel evidence of the chondroprotective actions of CS derived from shark and porcine trachea cartilages on human articular chondrocytes and synovial fibroblasts: i.e., they effectively interfere with interleukin (IL)-1β-stimulated ADAMTS-4 and -5 gene expression in human articular chondrocytes and synovial fi-
broblasts in addition to the recovery of IL-1β-mediated suppression of aggrecan core protein mRNA. We also demonstrate that porcine cartilage-derived CS specifically enhances TIMP-1 production in human synovial fibroblasts and recovered with the IL-1β-mediated suppression of TIMP-3 mRNA in human articular chondrocytes.

MATERIALS AND METHODS

Materials
Chondroitin sulfate SC (CS-SC) with a molecular weight of 113 kDa was isolated from shark cartilage, and it contains mainly chondroitin-6-sulfate (C6S) (69.3%) and chondroitin-4-sulfate (C4S) (17.8%), respectively (Table 1). The analysis for the property of those CS was performed as previously described.15)

Cell Culture
Normal human articular chondrocytes were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, U.S.A.). Chondrocytes were encapsulated in alginate beads and cultured in Dulbecco’s modified Eagle’s medium (DMEM)-F12 (Invitrogen, Co., Carlsbad, CA, U.S.A.)/10% fetal bovine serum (FBS) (Thermo ELECTRON Co., Melbourne, Australia) as previously described.16)

Briefly, chondrocytes were suspended in 1.2% alginate/0.15 M NaCl at the density of 4 x 10⁶ cells/ml, and the suspension was dropped into 102 mM CaCl₂ solution under gentle stirring to form the alginate beads. The beads (approximately 4 x 10⁴ cells/bead) were washed several times in 0.15 M NaCl, and then equilibrated with DMEM-F12/10% FBS. The cells embedded in alginate beads were cultured in DMEM-F12/10% FBS with 50 μM ascorbic acid-2-phosphate in 24-well plates (4 beads/well) for one week, and then cells were treated with IL-1β (10 ng/ml) and CS-SC or CS-PC (1, 10, 100 μg/ml) in DMEM-F12/0.2% lactalbumin hydrolysate (LAH) (Sigma Chemical Co., St. Louis, MO, U.S.A.) with 50 μM ascorbic acid-2-phosphate for 6 d. The culture medium was changed every 3 d.

Normal human synovial fibroblasts (Cell Systems Biotechnologie Vertrieb GmbH, St. Katharinen, Germany) were cultured in DMEM (Invitrogen Co.),/10% FBS until confluence. Synovial fibroblasts up to the 10th passage were seeded in 60-mm diameter culture dishes or 12-well plates. After confluence, cells were treated with IL-1β (10 ng/ml) and CS-SC or CS-PC (1, 10, 100 μg/ml) in DMEM/0.2% LAH. The harvested culture medium was stored at −20°C until use.

RNA Extraction and Quantitative Real-Time RT-PCR
Total RNA was extracted from the cells using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer’s instructions. Total RNA (1 μg) was subjected to reverse transcription (RT) using QuantiTect Reverse Transcription Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer’s instructions. A portion (equivalent to 2.5 ng of total RNA) of the products of RT was subjected to real-time polymerase chain reaction (PCR) using QuantiTect SYBR Green PCR Kit (Qiagen K.K.) and QuantiTect Primer Assays [Cat No. QT00032949 for human ADAMTS-4, Cat No. QT00011088 for human ADAMTS-5, Cat No. QT0001764 for human MMP-13, Cat No. QT00084168 for human TIMP-1, QT00046382 for human TIMP-3, and QT00079247 for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Qiagen K.K.] PCR was performed using ABI PRISM 7000 sequence detection system (Applied Biosystems Japan, Ltd., Tokyo, Japan) under the following conditions, denaturation for 15 s, annealing for 30 s, and extension for 30 s.

Western Blot Analysis
The protein level of TIMP-1 in culture medium was determined by Western blot analysis as described previously.13) Briefly, the proteins separated by SDS-PAGE were electro-transferred onto a nitrocellulose membrane filter, and the filter was reacted with sheep anti-human TIMP-1 antiserum (kindly provided by Prof. Hideaki Nagase, Kennedy Institute of Rheumatology, Imperial College London) that was then complexed with horseradish peroxidase-conjugated goat anti-(sheep IgG) IgG (Sigma Chemical Co.). Immunoreactive TIMP-1 bands were detected by enhanced chemiluminescence (ECL)-Western blotting detection reagents (GE Healthcare UK Ltd., Buckinghamshire, U.K.) and LAS-1000 plus (Fujifilm Co., Tokyo, Japan). Detected bands were quantitated using computer software Image Gauge version 3.41 (Fujifilm Co.).

Statistical Analysis
One-way ANOVA was performed using computer software StatView version 5.0 (SAS Institute, Inc., SAS Campus Drive Cary, NC, U.S.A.) for the data analysis. Independent Student’s t-test was applied for pair comparisons, and Fisher’s PLSD post-hoc test was performed for multiple comparisons. The level of statistically significant difference was set at p<0.05.

RESULTS

CS Interfered with IL-1β-Mediated ADAMTS-4 and -5 Gene Expression and Augmented the Expression of Aggrecan Core Protein mRNA in Human Articular Chondrocytes We examined the effects of CS-SC and CS-PC on the gene expression of ADAMTS-4 and -5 in cultured human articular chondrocytes. After one-week subculture chondrocytes in alginate beads were treated with IL-1β and CS-SC or CS-PC for 6 d. Results are shown in Fig. 1. Although both CS-SC and CS-PC suppressed IL-1β-enhanced expression of ADAMTS-4 and -5 mRNA in human articular chondrocytes,

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<th>Table 1. Disaccharide Composition of CS-SC and CS-PC</th>
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ΔDi-0S, chondroitin; ΔDi-4S, chondroitin-4-sulfate (C4S); ΔDi-6S, chondroitin-6-sulfate (C6S); ΔDi-diS₄, chondroitin-4,6-disulfate; ΔDi-diS₆, chondroitin-2,4-disulfate; ΔDi-diS₈, chondroitin-2,6-disulfate; ΔDi-triS, chondroitin-2,4,6-trisulfate; and N.D., not detected.
no obvious dose-dependent suppression was seen with either CS; the most effective concentration was 10 μg/ml for both CS. In contrast, CS-SC and CS-PC recovered the IL-1β-mediated suppression of aggrecan core protein mRNA in a dose-dependent manner (Fig. 2). While expression of interstitial collagenase/MMP-1 and stromelysin-1/MMP-3 was not modulated by CS-SC and CS-PC (data not shown), both CS interfered with IL-1β-mediated MMP-13 gene expression at the doses of 1 and 10 μg/ml (Fig. 2).

CS-SC and CS-PC modulated the expression of TIMPs in human articular chondrocytes, which constitutively expressed mRNA of TIMPs-2 and -3, and also slightly expressed TIMP-1. CS-SC and CS-PC did not affect the expression of TIMPs-1 and -2 (data not shown). CS-PC, but not CS-SC effectively recovered the IL-1β-mediated suppression of TIMP-3 mRNA in a dose-dependent manner (Fig. 2).

CS Also Interfered with the Expression of IL-1β-Mediated ADAMTS-4 and -5 mRNA in Human Synovial Fibroblasts Since synovial fibroblasts produce ADAMTS-4 and -5, the same as articular chondrocytes, we examined the effects of CS on the gene expression of these enzymes in cultured human synovial fibroblasts. The synovial fibroblasts were co-treated with IL-1β and CS-SC or CS-PC for 24 h, and the expression of ADAMTS-4 and -5 was evaluated by real-time RT-PCR. The expression of both aggrecanases was significantly increased by IL-1β (Fig. 3). Both CS-SC and CS-PC effectively interfered with IL-1β-enhanced ADAMTS-4 and -5 gene expression in human synovial fibroblasts already demonstrated in articular chondrocytes (Fig. 3), whereas MMPs-1 and -3 expression was not affected by these CS (data not shown).

Unlike articular chondrocytes, human synovial fibroblasts predominantly secrete TIMP-1, which controls MMP activity. When human synovial fibroblasts were treated with CS-PC for 4 d, TIMP-1 in the culture medium was increased in a dose-dependent manner (Figs. 4A, B). CS-SC also enhanced TIMP-1 production but the increase was not statistically significant (Figs. 4A, B). In contrast, neither CS-SC nor CS-PC modulated TIMP-1 mRNA in human synovial fibroblasts at 24 h (data not shown) nor at 4 d (Fig. 4C). These results suggest that both CS-SC and CS-PC promote the production of TIMP-1 in human synovial fibroblasts at a post-transcriptional level.

DISCUSSION

Although natural CS of different origins is used worldwide to treat arthritic diseases such as OA, its anti-arthritic action mechanisms have not been clarified. CS has been reported to decrease IL-1β-induced gene expression of MMPs-1, -3, and -13, and ADAMTS-4 and -5 in bovine chondrocytes cultured in alginate beads under hypoxic (5% O2) conditions.10 While
and -5 mRNA expression, because IL-1β is one of the most prominent and potent catabolic factors in OA cartilage.\(^{21,22}\)

In addition, CS has been reported not to bind to IL-1β.\(^{23}\) Thus, the effects of CS on the expression of MMPs and aggrecanases vary widely from report to report,\(^{18—20}\) probably due to the cell species used, culture conditions, and the origin of CS.

Since CS was first applied to recover articular function, most attention has been paid on how it exerts its effects on articular chondrocytes. In the present study, we first examined the effects of CS-SC and CS-PC on the expression of aggrecanases in human articular chondrocytes and synovial fibroblasts. IL-1β was used as a stimulant in ADAMTS-4 and -5 mRNA expression, because IL-1β is one of the most prominent and potent catabolic factors in OA cartilage.\(^{21,22}\)

In addition, CS has been reported not to bind to IL-1β.\(^{23}\) Both CS-SC and CS-PC effectively interfered with the IL-1β-enhanced gene expression of ADAMTS-4 and -5 in human articular chondrocytes and synovial fibroblasts. Messenger RNA levels of ADAMTS-4 and -5 well correlate with their enzymatic activities,\(^{24,25}\) therefore CS-SC and CS-PC are likely to attenuate the aggrecanase-mediated destruction of articular cartilage in OA. However, no dose-dependent suppression was observed, and at a low concentration CS-SC and CS-PC suppressed ADAMTS-4 and -5 mRNA expression more effectively than at the highest concentration used in this study. Similar effects were observed with MMP-13 expression, and thus the optimal concentration of CS-SC and CS-PC to suppress gene expression of ADAMTS-4 and -5, and MMP-13 was about 10 μg/ml. On the other hand, the expression of aggrecan core protein, which is suppressed by IL-1β, was significantly recovered by 100 μg/ml of CS-SC and CS-PC. Taken together, these results suggest that at the low concentration (ca. 10 μg/ml) CS interfered with enzymatic cartilage degradation, while at the highest concentration (ca. 100 μg/ml) it accelerated ECM anabolism in the articular cartilage. In contrast, we could not identify the precise mechanisms of the suppression of CS-SC and CS-PC on gene expression and the production of MMPs-1 and -3, unlike as previously reported.\(^{18,20}\) There might be less or no effect of CS-SC and CS-PC on MMPs-1 and -3 production in human articular chondrocytes and synovial fibroblasts.

Furthermore, we have provided novel evidence that IL-1β-reduced expression of TIMP-3 mRNA was recovered by 100 μg/ml of CS-SC and CS-PC. Taken together, these results suggest that the low concentration (ca. 10 μg/ml) CS interfered with enzymatic cartilage degradation, while at the highest concentration (ca. 100 μg/ml) it accelerated ECM anabolism in the articular cartilage. In contrast, we could not identify the precise mechanisms of the suppression of CS-SC and CS-PC on gene expression and the production of MMPs-1 and -3, unlike as previously reported.\(^{18,20}\) There might be less or no effect of CS-SC and CS-PC on MMPs-1 and -3 production in human articular chondrocytes and synovial fibroblasts.
actions in the articular cartilage. Moreover, CS-PC significantly increased TIMP-1 production without affecting its mRNA expression in human synovial fibroblasts, suggesting that CS-PC up-regulates the production of TIMP-1 at a post-transcriptional level. CS-SC also increased TIMP-1 production but to a lesser extent. Since CS-PC mainly consists of C4S (72.8%) and CS-SC mostly contains C6S (69.3%), the compositional distinction is considered to make a difference of their functions. Regarding the different actions of CS isoforms, Campo et al.28) reported that intradermally injected C4S suppressed nuclear factor (NF)-κB activation in mice with collagen-induced arthritis. Furthermore, the transfection of TIMP-3 has been reported to inhibit TNFα-induced activation of NF-κB in synovial fibroblasts29), therefore, the increase of TIMP-3 expression by C4S may suppress NF-κB activation in arthritic mice. On the other hand, C6S has been reported to exert anti-arthritic action in mice with collagen-induced arthritis30) and in rats with bradykinin-induced proteoglycan depletion.31) Thus, both C6S and C4S would exert an anti-arthritic action through mutual and individual mechanisms.

Recent meta-analyses of the treatment of osteoarthritis by CS revealed that CS has slight to moderate efficacy or non-existent efficacy against pain due to OA32,33). During the preparation of this paper, it was reported that long-term application of C4S and C6S improve symptoms and delay joint preparation of this paper, it was reported that long-term treatment. Thus, moderate efficacy with extremely lower side effects characterizes the preventive action of CS on cartilage destruction.

In conclusion, both CS-SC and CS-PC prevent cartilage destruction due to the suppression of gene expression of ADAMTS-4 and -5, and MMP-13, and promote cartilage remodeling through the up-regulation of aggrecan core protein expression. In addition, CS-PC increases the TIMP-1 production and TIMP-3 mRNA, resulting in further inhibitory effect on MMPs and aggrecanase activities. Furthermore, we provide the first evidence that some of the above actions of CS are also exerted in synovial fibroblasts. Therefore, CS probably modulates functions of both chondrocytes and synovial fibroblasts, and thereby exerts multifunctional chondroprotective action in degenerative arthritic diseases including OA.

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REFERENCES