学　位　論　文

Sulfation patterns of exogenous chondroitin sulfate affect chondrogenic differentiation of ATDC5 cells

(コンドロイチン硫酸の硫酸化構造が軟骨前駆細胞株 ATDC5 の分化に及ぼす影響)

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ORIGINAL ARTICLE

Sulfation patterns of exogenous chondroitin sulfate affect chondrogenic differentiation of ATDC5 cells

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Abstract
Background Chondroitin sulfate (CS) has been used in cartilage tissue engineering techniques as a positive modulator of scaffolds. CS is a linear polysaccharide consisting of variously sulfated repeating disaccharides. The sulfation patterns of CS are closely related to their biological functions, but only monosulfated CS has been applied to scaffolds. In this study, we investigated the effects of various sulfation patterns of CS on chondrogenic differentiation using ATDC5 chondroprogenitor cells.
Methods Disaccharide composition analysis of CS produced by ATDC5 cells at various differentiation steps was performed using high-performance liquid chromatography. ATDC5 cells were cultured with exogenously added, variously sulfated CS. Cell proliferation was analyzed by the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay. Extracellular matrix production was evaluated by Alcian blue staining. Alkaline phosphatase (ALP) activity was evaluated using an ALP assay kit. Expression of chondrogenic markers was evaluated by real-time reverse transcription polymerase chain reaction (RT-PCR) or an enzyme-linked immunosorbent assay (ELISA) using a Type II Collagen Detection kit.
Results The major components of CS produced by ATDC5 cells were 4-O-monosulfated disaccharides throughout chondrogenic differentiation. Low proportions of 4,6-O-disulfated disaccharides were also detected. Compared to the control group, which did not contain GAGs, the WST-8 assay indicated fewer viable cells when treated with CS-E, which are rich in 4,6-O-disulfated disaccharides. CS-E significantly enhanced Alcian blue staining in a dose-dependent manner and decreased ALP activity after 21 days of culture. Real-time RT-PCR showed that CS-E significantly enhanced all chondrogenic markers, col2a1, aggrecan, and sox9, either at day 4 or day 14 of culture. The results of ELISA analysis confirmed that CS-E significantly enhanced the production of type II collagen.
Conclusions ATDC5 cells produced four different monosulfated or disulfated disaccharides in their extracellular matrices. The sulfation patterns of exogenously added CS affected chondrogenic differentiation of ATDC5 cells. In particular, CS-E rich in disulfated disaccharides significantly promoted chondrogenic differentiation of ATDC5 cells. Thus, CS containing this disulfated structure may be a useful scaffold component for enhancing chondrogenesis in cartilage tissue engineering.

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Introduction

Articular cartilage tissue has very little capacity for self-repair because of its avascular nature and paucity of chondrocytes. Once articular cartilage undergoes severe damage, irreversible degeneration of the cartilage tissue occurs and leads to osteoarthritis, which adversely affects quality

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of life. Tissue engineering is one possible means of treating severe injuries in articular cartilage [1].

Scaffolds are quite important for achieving hyaline-like cartilage regeneration in tissue engineering. Many studies have been conducted with the aim of developing effective scaffolds [2]. Moreover, various molecules have been applied to scaffolds to try to modify them and create a suitable microenvironment in which cells can differentiate properly and maintain chondrogenic properties [3]. There have been some successful modifications with glycosaminoglycans (GAGs), such as hyaluronan, heparan sulfate, or chondroitin sulfate (CS) [3]. Of these GAGs, CS, a major component of extracellular matrices (ECMs) in cartilage tissue, is a favored candidate biomaterial for an effective scaffold modifier [4–6].

CS is an unbranched linear polysaccharide consisting of repeating sulfated disaccharide units containing D-glucuronic acid and *N*-acetyl-D-galactosamine. CS chains are ubiquitously found as GAG side chains of proteoglycans in the ECM and at cell surfaces. Ordinary CS chains contain 4-*O*- and 6-*O*-monosulfated disaccharide units in various proportions, and are designated as CS-A or CS-C depending on the ratio of the building units. Other disaccharide structures in CS include disulfated and highly sulfated disaccharides. CS chains rich in over sulfated 2,6-*O*-disulfated disaccharide units or 4,6-*O*-disulfated disaccharide units are designated as CS-D or CS-E (Fig. 1); [7]. Recent studies have suggested that CS has many biological roles, such as signaling functions of various growth factors, involvement in the development of the central nervous system, and receptor functions for various pathogens [8]. These functions are closely associated with the sulfation patterns of the CS moieties. Moreover, mutations in the genes of sulfotransferases, active sulfate synthetase or nucleotide-sugar transporters, which are indispensable for the synthesis of CS, cause chondrodysplasia in mice and humans [9–14]. These findings confirm that the sulfation of CS plays a critical role in chondrogenic differentiation.

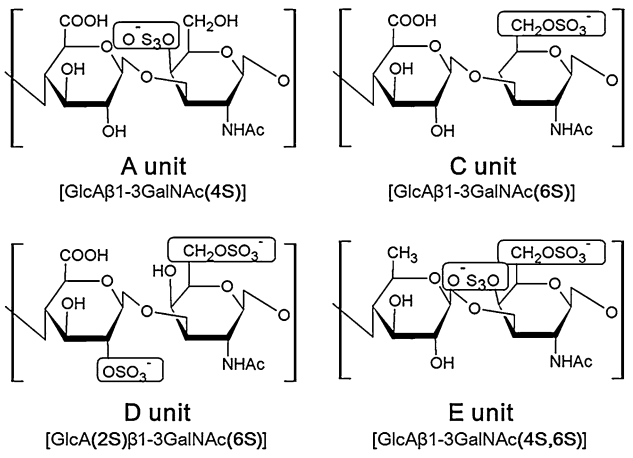

Fig. 1 Four sulfated disaccharide structures identified in various chondroitin sulfate chains. *GlcA* D-glucuronic acid, *GalNAc N*-acetyl-D-galactosamine

In this study, we hypothesized that the sulfation patterns of exogenously added CS would affect the chondrogenic differentiation of ATDC5 chondroprogenitor cells. ATDC5 is a prechondrogenic stem cell line originally isolated from a mouse embryonal carcinoma. ATDC5 cells provide an excellent in vitro model system that mimics the multistep process of chondrogenic differentiation [15]. The aims of this study were (1) to confirm the sulfation patterns of the disaccharide components of CS produced by ATDC5 cells at various differentiation steps, and (2) to evaluate possible effects of CS with various structures on the proliferation or chondrogenic differentiation of ATDC5 cells. The results obtained here will help to improve the modification of scaffolds to induce more effective chondrogenic differentiation.

Materials and methods

Reagents

CS-A from whale cartilage, CS-C from shark cartilage, CS-E from squid cartilage, and chondroitinase (CSase) ABC from *Proteus vulgaris* were purchased from Seikagaku Corp. (Tokyo, Japan). Holo-transferrin, sodium selenite, bovine insulin, elastase from porcine pancreas and pepsin from porcine gastric mucosa were purchased from Sigma (St. Louis, MO). Actinase E was obtained from Kaken Pharmaceutical Co. (Tokyo, Japan). BCA protein assay and alkaline phosphatase (ALP) activity assay kits (LABOASSAY™ ALP) were purchased from Thermo Fisher Scientific Inc. (Rockford, IL) and Wako (Osaka, Japan), respectively. The Type II Collagen Detection Kit was purchased from Chondlex Inc. (Redmond, WA).

Cell culture conditions

The ATDC5 cell line was obtained from Riken Cell Bank (Tsukuba, Japan). Cells were cultured in maintenance medium consisting of Dulbecco's modified Eagle's medium/Ham's F12 hybrid medium containing 5 % fetal bovine serum, 10 μg/mL holo-transferrin, and 3×10^{-8} M sodium selenite, at 37 °C in air with 5 % CO_2. After the cells reached confluence, the medium was supplemented with 10 μg/mL bovine insulin to induce chondrogenic differentiation. In some experiments, the maintenance medium was supplemented with insulin and various commercially available GAGs at a final concentration of 10 or 100 μg/mL. These two concentrations were optimized based on the results of preliminary experiments. The GAGs used in this study were CS-A, CS-C, and CS-E, which are rich in A (GlcAβ1-3GalNAc(4-*O*-sulfate)), C

(GlcAβ1-3GalNAc(6S), and E (GlcAβ1-3GalNAc(4S, 6S)) units, respectively. The culture medium was replaced every 2–3 days, and each GAG was added separately after replacement of the medium. The inoculum size of the cells was approximately 6,000–10,000 cells/cm^2, according to a report by Shukunami et al. [16] Cells cultured without GAGs were defined as the control group.

Extraction of GAGs from ATDC5 cells

GAGs produced by ATDC5 cells were extracted on days 0, 7 and 21 of culture. Cells were dehydrated and delipidated by extraction with acetone and air-dried. Each acetone powder preparation was weighed, digested with heat-activated actinase E, and then treated with 5 % trichloroacetic acid to deactivate the enzyme. The trichloroacetic acid was removed with diethyl ether. The aqueous phase was adjusted to 80 % ethanol and 1 % sodium acetate and kept at 4 °C overnight. The precipitated crude GAGs were desalted using an Amicon Ultra-4 (Merck Millipore, Billerica, MA) [17].

Disaccharide composition analysis

The extracted GAGs were digested with chondroitinase ABC and labeled with 2-aminobenzamide (2AB). The 2AB-labeled digest was subjected to anion-exchange high-performance liquid chromatography (HPLC) on a PA-03 silica column (YMC Co. Ltd., Kyoto, Japan). The resulting disaccharides were identified and quantified by comparison with the elution of authentic CS-derived unsaturated disaccharides [18].

Cell proliferation assay

Cell proliferation with or without GAGs was assayed at day 4, 7 and 21 after induction of the differentiation. Cell proliferation was determined by the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) assay using a Cell Counting Kit-8 (Dojindo Co., Kumamoto, Japan). The WST-8 assay is a colorimetric assay for the quantification of cell proliferation based on cleavage of WST-8 by mitochondrial dehydrogenases in viable cells. A 20 μL solution of WST-8 was added to each well of a 96-well plate, which was then incubated for 1 h at 37 °C. Absorbance at 450 nm was measured using a microplate spectrophotometer (Benchmark Plus™, Bio-Rad Laboratories, Hercules, CA). A total of eight samples were analyzed for each group.

ECM production

Sulfated GAGs produced by ATDC5 cells were assessed by staining with Alcian blue. After 21 days of culture, cells were fixed with 100 % methanol for 10 min at −20 °C and stained with Alcian blue solution, pH 2.5 (Wako) for 2 h at room temperature. To quantify the intensity of the staining, the stained cultures were extracted with 6 M guanidine/HCl. The optical density of the extracted dye was measured at 650 nm [19].

ALP activity

After 21 days of culture, cell lysates were collected in 0.1 % Triton X and centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatants were subjected to an ALP activity assay kit. Briefly, each sample, diluted 16-fold in water, was added into a 96-well plate and mixed with the substrate buffer in each well. After a 15 min incubation at 37 °C, 0.2 M sodium hydroxide was added to terminate the reaction. Absorbance of the samples was measured at 405 nm with a microplate spectrophotometer. Protein concentrations of cell lysates were measured by a BCA protein assay kit, according to the manufacturer's protocol. Enzyme activity was determined as units/μg protein. One unit of enzyme activity was defined as a release of 1 nmol *p*-nitrophenol per min at pH 9.8 and 37 °C.

RNA isolation and real-time reverse transcription polymerase chain reaction (RT-PCR)

Real-time RT-PCR was used to assess the expression of the chondrogenic markers collagen type II, aggrecan, and Sox9 at days 4 and 14, and collagen type X at day 14 after cultivation. Total RNA was extracted from the cultured cells with Trizol reagent (Invitrogen Corp., Carlsbad, CA) and 0.5 μg of the extracted RNA was used for cDNA synthesis using ImProm-II reverse transcriptase (Promega, Madison, WI) and random primers (Promega). Quantitative analysis of mRNA levels was performed with Opticon Real-Time PCR (Bio-Rad Laboratories, Hercules, CA) using sequence-specific primer pairs (Table 1). Primers were designed with mouse sequences using Oligo software (Molecular Biology Insights, Cascade, CO). PCR was carried out using SYBR Green Master Mix (Finnzymes, Espoo, Finland) according to the manufacturer's protocol. All real-time PCR reactions were performed in triplicate and the levels of mRNA expression were calculated and normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA at each time point.

Quantification of collagen type II

Collagen type II produced by ATDC5 cells was quantified by an enzyme-linked immunosorbent assay (ELISA) using a Type II Collagen Detection kit. After 14 days of culture, cells were washed with PBS and 0.05 M acetic acid was added to the cell layer. Cells were harvested by scraping and transferred to microcentrifuge tubes. A solution of 0.01 % pepsin in 0.05 M acetic acid was added to the sample tubes, and the

Table 1 Real-time polymerase chain reaction primers used in the experiments

Target gene	Forward primer	Reverse primer
Col2a1	5′-AGGATGGCTGCACGAAACAC-3′	5′-TGTCCATGGGTGCGATGTC-3′
Col10a1	5′-GGCTTCAGGGAGTGCAATC-3′	5′-CTCACATGGGAGCCACTAGG-3′
Aggrecan	5′-CCCTCACCCCAAGAATCAAG-3′	5′-GGATAGTTGGGGAGCGACAC-3′
Sox9	5′-CCCCGATTACAAGTACCAGC-3′	5′-AGCGCCTTGAAGATAGCATT-3′
GAPDH	5′-GTCGGTGTGAACGGATTTGG-3′	5′-CAATGAAGGGGTCGTTGATG-3′

GAPDH glyceraldehyde 3-phosphate dehydrogenase

Table 2 Composition of disaccharides produced in extracellular matrices by ATDC5 cells

	ΔO	ΔA	ΔC	ΔD	ΔE	Total amount
	pmol/mg acetone powder (mol % in total disaccharides)					
Day 0	2.1	22.1	0.2	ND	0.4	24.9
	(8.4)	(88.9)	(1.0)		(1.7)	
Day 7	4.4	37.5	0.6	ND	0.6	43.2
	(10.3)	(86.8)	(1.5)		(1.4)	
Day 21	242.8	940.2	47.1	ND	8.7	1,238.8
	(19.6)	(75.9)	(3.8)		(0.7)	

ΔO ΔHexUAα1–3GalNAc, *ΔA* ΔHexUAα1–3GalNAc(4-*O*-sulfate), *ΔC* ΔHexUAα1–3GalNAc(6-*O*-sulfate), *ΔD* ΔHexUA(2-*O*-sulfate)α1–3GalNAc(6-*O*-sulfate), *ΔE* ΔHexUAα1–3GalNAc(4,6-*O*-disulfate), *HexUA* 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid, *GalNAc* *N*-acetylgalactosamine, *ND* not detected

cell suspension was digested for 24 h at 4 °C on a rocker. After the sample tubes were centrifuged at 10,000 rpm for 3 min, the supernatants were collected and transferred to new collection tubes. The pepsin digestion was repeated three times for each sample, and 0.01 % pancreatic elastase in Tris-buffered saline (pH 7.8) was finally added into the sample tubes. The sample tubes were incubated for 24 h at 4 °C and then centrifuged at 10,000 rpm for 3 min. The resulting supernatants were collected into collection tubes and the sample volumes were adjusted with sample dilution buffer. Collagen type II ELISA was conducted according to the manufacturer's protocol. A total of four samples was analyzed for each group.

Statistical analysis

All results are expressed as means and standard deviation. Statistical analysis was performed by analysis of variance using Dunnett's method for multiple comparisons. p values of less than 0.05 were considered significant.

Results

Disaccharide composition analysis

The total amount of disaccharides produced by ATDC5 cells increased during their differentiation. The major component of chondroitin sulfate was 4-*O*-monosulfated disaccharide, ΔA, throughout chondrogenic differentiation. Although detected, the proportion of 4,6-*O*-disulfated disaccharides was low (Table 2).

Cell proliferation assay

Compared to the control group, fewer viable cells were detected in the WST-8 assay after being treated with CS-E at days 7 and 14 of culture. However, no statistical difference in cell viability was found between CS-E treated and control cells at day 4 of culture (Fig. 2).

ECM production

CS-E significantly enhanced Alcian blue staining compared with the control group. Moreover, CS-E dose-dependently enhanced staining after days 21 of culture. The other examined GAGs showed a slight dose-dependent decrease in Alcian blue staining. The decrease in Alcian blue staining induced by exogenously added CS-C at a concentration of 100 μg/mL reached the level of statistical significance (Fig. 3). Blank culture wells incubated with GAGs for 3 weeks showed no Alcian blue staining in a preliminary negative control experiment (data not shown).

ALP activity assay

CS-E (100 μg/mL) significantly decreased ALP activity after 21 days of culture, whereas the other examined GAGs did not affect enzymatic activity (Fig. 4).

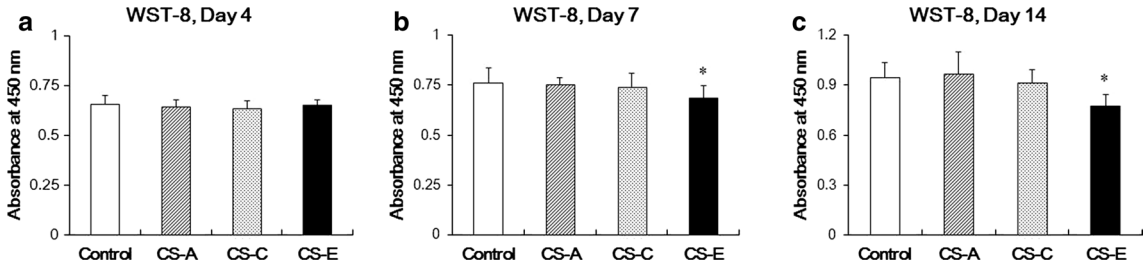

Fig. 2 Cell proliferation assay at day 4 of culture showed no statistical differences between GAGs (**a**). At days 7 and 21 of culture, CS-E significantly decreased ATDC5 cell proliferation (**b**, **c**) ($n = 8$), * $p < 0.01$

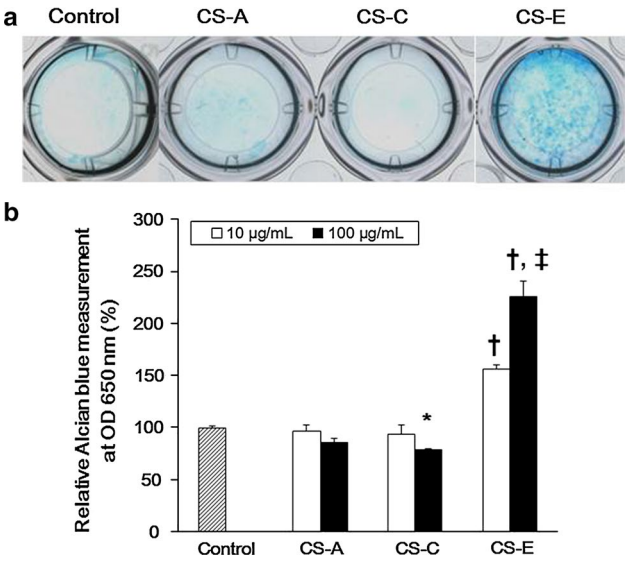

Fig. 3 **a** After 21 days of culture, sulfated GAGs produced by ATDC5 cells were stained with *Alcian blue* dye. **b** The stained cultures were extracted with 6 M guanidine/HCl and quantified ($n = 3$), $^{*}p < 0.05$ vs. control group, $^{\dagger}p < 0.001$ vs. control group, and $^{\ddagger}p < 0.001$ when compared among doses. *CS-A* chondroitin sulfate A, *CS-C* chondroitin sulfate C, *CS-D* chondroitin sulfate D, *CS-E* chondroitin sulfate E, *OD* optical density

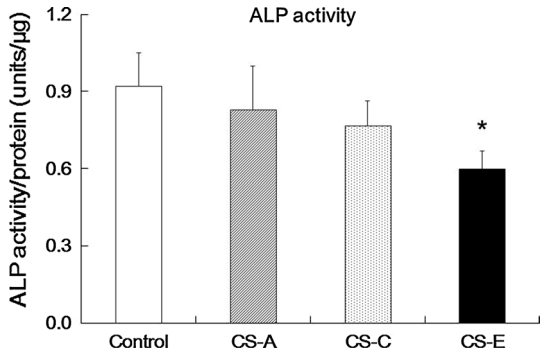

Fig. 4 Alkaline phosphatase (ALP) activity was measured using an assay kit after 21 days of culture. Protein concentrations of the cell lysates were measured using a BCA protein assay kit. Enzyme activity is reported as units/μg protein ($n = 4$), $^{*}p < 0.01$

Chondrogenic differentiation

Consistent with the increase in Alcian blue staining in CS-E-treated ATDC5 cells, real-time RT-PCR showed that CS-E significantly enhanced the expression of the chondrogenic marker genes *col2a1*, *aggrecan,* and *sox*9 by days 4 and 14 of culture. In contrast, the other GAGs downregulated all tested marker genes. However, among the examined marker genes, only the change in expression of *col10a1* reached the level of statistical significance (Fig. 5). The expression of collagen type II at the protein level was confirmed by ELISA. CS-E treatment enhanced the production of collagen type II and showed the same tendency as the results of RT-PCR (Fig. 6). The other GAGs did not affect the production of collagen type II.

Discussion

We investigated whether the sulfation patterns of CS affect the chondrogenic differentiation of ATDC5 cells. Disaccharide composition analysis revealed that ATDC5 cells produced four different disaccharide units, namely, nonsulfated, 4-*O*-monosulfated or 6-*O*-monosulfated, and 4,6-*O*-disulfated disaccharides. The rich 4,6-*O*-disulfated structure of CS-E significantly enhanced Alcian blue staining and upregulated the expression of chondrogenic marker genes throughout the chondrogenic differentiation of ATDC5 cells. CS-E significantly decreased cell proliferation and ALP activity. Neither CS-A nor CS-C showed any significant effects on the proliferation or differentiation of ATDC5. Taken together, these results indicate that the sulfation patterns of CS, particularly CS-E, positively affect the chondrogenic differentiation and maintenance of the chondrogenic phenotype of ATDC5 cells.

In normal human articular cartilage, the main disaccharide components are 4-*O*-monosulfated or 6-*O*-monosulfated and nonsulfated disaccharides. Disulfated or higher sulfated structures are present, but they account for less than 1 % of the total disaccharides [20]. Disulfated disaccharides are also reported to be present in

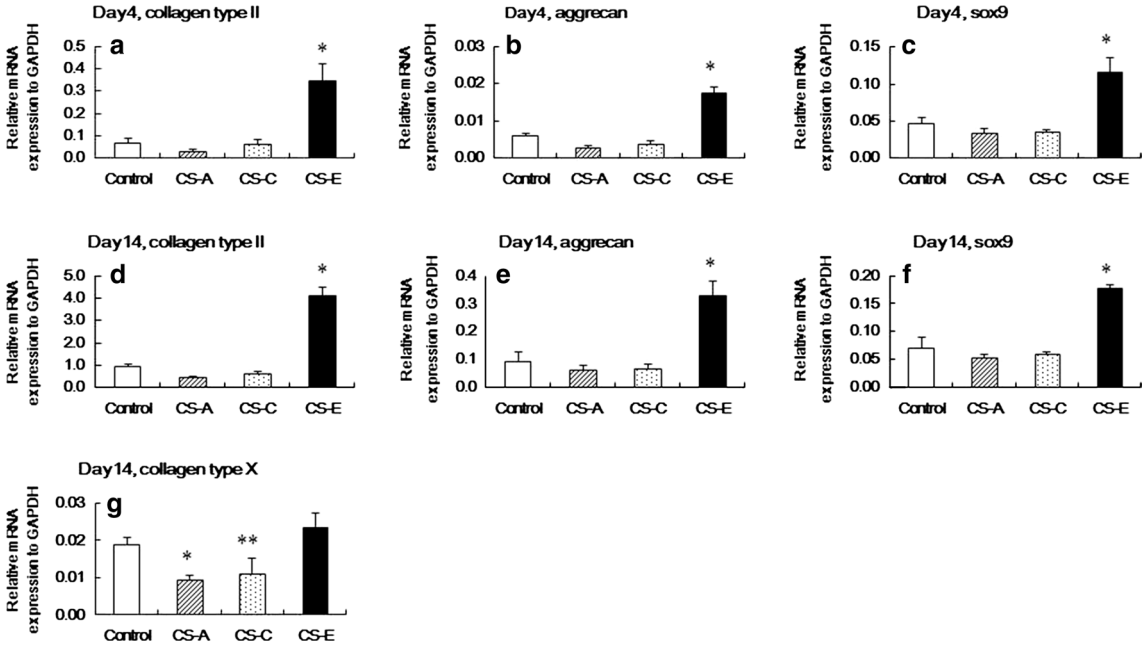

Fig. 5 Expression of chondrogenic markers by ATDC5 cells was evaluated by RT-PCR at days 4 (**a**–**c**) and 14 (**d**–**g**) of culture. **a–f** CS-E significantly upregulated all chondrogenic markers tested at days 4 and 14. **g** CS-A and CS-C significantly suppressed the expression of collagen type X gene, which is expressed in hypertrophic cells, at day 14, whereas CS-E did not ($n = 3$) CS, chondroitin sulfate, $*p < 0.01$ vs. control, $**p < 0.05$ vs. control

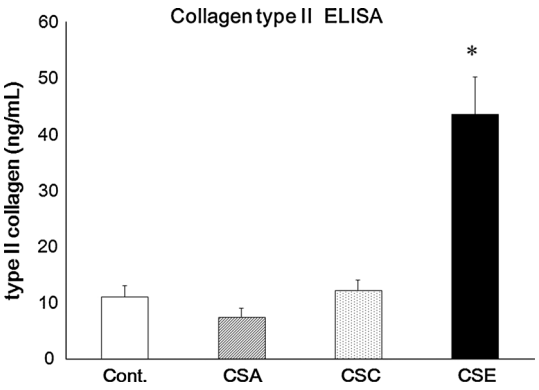

Fig. 6 Quantification of collagen type II protein produced by ATDC5 cells at day 14 of culture. CS-E significantly enhanced collagen type II production ($n = 4$), $*p < 0.01$

rat, chicken, and bovine [7, 21, 22]. In this study, while the main components were 4-*O*-sulfated disaccharides (> 70 %), a small amount of 4,6-*O*-disulfated disaccharides (0.5–1.7 %) was produced by ATDC5 cells throughout the 3 weeks of the differentiation course. These results, together with those showing that CS-E promoted chondrogenic differentiation of ATDC5, indicate that 4,6-*O*-disulfated disaccharides in CS polysaccharides may play an important role in the acceleration of cartilage tissue differentiation.

Chondrogenic marker gene expressions and ECM production were significantly enhanced when ATDC5 cells were cultured with CS-E. Recent studies have shown various biological functions of CS in addition to their conventional structural roles, such as water absorption due to negative charge [7]. Among the CS variants, CS-E and CS-D, which are highly sulfated, promote the outgrowth of neurites in embryonic mouse hippocampal neurons [23]. These two CS variants also show strong affinity to some growth factors, such as fibroblast growth factors and bone morphogenetic proteins (BMPs) [24, 25]. CS-E enhances osteoblast differentiation by binding to BMP-4, which is an essential growth factor for chondrogenic differentiation [26]. ATDC5 cells express the *BMP-4* gene and autocrine BMP-4 is required for the differentiation of ATDC5 [27]. Therefore, it is possible that CS-E enhanced the differentiation of ATDC5 cells by binding to endogenously produced BMP-4 molecules, thereby prolonging and stabilizing the biological activity of BMP-4. BMP-4 is reported to induce ALP activity in ATDC5 cells [28]; however, CS-E reduced the ALP activity of cells in the present study (Fig. 4). As the induction of ALP activity occurred at a later phase of differentiation (day 42 of culture), our findings suggest that BMP-4 does not induce ALP activity during the early differentiation phase in the first 21 days of culture. A number of growth factors other than BMP-4 are also able to bind CS-E and may have contributed to the positive effects of

CS-E on the chondrogenic differentiation of ATDC5 cells. Further studies are therefore required to elucidate the mechanisms of chondrogenic differentiation promoted by CS-E.

In tissue engineering techniques, three important factors—cell sources, scaffolds, and signals—play key roles in the successful regeneration of tissues [29]. Thus, it is reasonable to apply specific growth factors to scaffolds for the purpose of enhancing differentiation steps. However, growth factors usually have short half-lives and are expensive, so it is not practical to continue treatment with a certain concentration of a particular growth factor until cartilage tissue regeneration is complete, especially in a clinical setting. CS moieties cross-linked to scaffolds may extend the half-lives of growth factors and provide prolonged activity by capturing the growth factors and recruiting them to particular receptors.

The application of CS-A and CS-C to scaffolds is reported to be effective for mesenchymal stem cell (MSC) chondrogenesis by providing suitable microenvironments [4, 6]. These CS variants were selected because they contain 4-*O*-sulfated or 6-*O*-sulfated structures, which are major components of CS in normal cartilage. CS-E is a more feasible candidate for scaffolds that can induce chondrogenic differentiation more effectively by interacting with endogenous growth factors.

There are several limitations to our study. First, because the results were obtained in an in vitro cell culture system, the effectiveness of CS-E observed in this study must be confirmed through in vivo experiments. Second, ATDC5 cells were isolated from a mouse teratocarcinoma stem cell line; thus, the sulfation patterns of CS would be different from those in human articular cartilage tissues. Finally, the mechanism of chondrogenic differentiation enhanced by CS-E was not elucidated in this study. As only CS-E contains a 4,6-*O*-disulfated structure, a disulfated structure seems to be a critical factor for inducing chondrogenic differentiation. Further studies are required to uncover the underlying mechanism.

In conclusion, ATDC5 cells produced variously sulfated disaccharide structures in their extracellular matrices. The sulfation patterns of exogenously added CS affected the chondrogenic differentiation of ATDC5 cells. In particular, CS-E, which is rich in disulfated disaccharides, significantly promoted chondrogenic differentiation of ATDC5 cells. Thus, CS polysaccharides with this structure are feasible components of scaffold material for enhancing the chondrogenesis of stem and progenitor cells, by modulating growth factors in cartilage tissue engineering.

Conflicts of interest None of the authors have any conflicts of interest to declare.

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