CHONDROITIN SULFATE N-ACETYLGLACTOSAMINYLTRANSFERASE-1 PLAYS A CRITICAL ROLE IN CHONDROITIN SULFATE SYNTHESIS IN CARTILAGE

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Running Title: CSGalNAcT-1 is critical for CS biosynthesis in cartilage

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Cartilage destruction leads to severe joint diseases such as osteoarthritis and spinal disorders with back pain and cartilage regeneration is very inefficient. A major component of the cartilage extracellular matrix is the proteoglycan aggrecan that contains approximately one hundred chondroitin sulfate (CS) chains, which impart water absorption and resistance to compression. Here, we demonstrate that chondroitin sulfate N-acetylgalactosaminylntransferase-1 (CSGalNAcT-1) plays a critical role in CS biosynthesis in cartilage. By in situ hybridization and real time RT-PCR of developing cartilage CSGalNAcT-1 exhibited the highest level of expression. Its expression in chondrogenic ATDC5 cells correlated well with that of aggrecan core protein. In heterozygote and homozygote aggrecan-null cartilage where aggrecan transcription is decreased, CSGalNAcT-1 transcription diminished accordingly. Overexpression of the enzyme in chondrocytic cells further enhanced CS biosynthesis but not that of the aggrecan core protein, indicating that the enzyme activity is not saturated in the cells and that aggrecan synthesized in the overexpressing cells is heavier than the native molecule. Analysis of the CS chains synthesized in the overexpressing cells by gel chromatography and that of disaccharide composition revealed that the CS chains had similar length and sulfation patterns. Furthermore, adenoviral gene delivery of the enzyme into intervertebral discs displayed a substantial increase in the level of CS biosynthesis. These observations indicate that CSGalNAcT-1 overexpression increases the number of CS chains attached to aggrecan core protein. Our studies may lead to a new therapeutic intervention, ameliorating the outcome of cartilage degenerative diseases.

Cartilage is localized on joint surfaces and in the spine where it forms the intervertebral discs and helps physical movement. The extracellular matrix of cartilage contains two major structural components: the fiber structure made of collagens and the proteoglycan aggregate. Whereas collagen fibers of types II, IX, and XI provide cartilage with tensile strength, the proteoglycan aggregate, composed of a large proteoglycan aggrecan, hyaluronan, and link protein, provides it with resistance to compression (1). Aggrecan consists of a core protein and approximately a hundred chains of chondroitin sulfate (CS) attached to the core protein (2, 3). Aggrecan is incorporated into the cartilage matrix by binding hyaluronan, and the CS chains impart water absorption. The CS content of aggrecan gradually diminishes with age, resulting in decreased water retention and aggravation of cartilage degeneration (4–6). This leads to severe joint diseases such as osteoarthritis (7, 8) and spinal disorders with back pain (9, 10). Due to the lack of blood vessels and the presence of specific structural macromolecules such as aggrecan, cartilage is one of the most difficult tissues to regenerate.

CS comprises repeating disaccharide units of N-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA) residues with sulfate residues at various positions. CS biosynthesis (11) (Fig. 1A) is initiated by the transfer of a GalNAc residue to the linkage region of a GlcA-β1,
3-galactose (Gal)-β1, 3-Gal-β1, 4-Xyl tetrasaccharide primer that is attached to a serine residue of the core protein. Then, chain elongation occurs by the alternate addition of GalNAc and GlcA residues. Enzyme activities that catalyze the initiation and elongation processes are termed glycosyltransferase-I and II activities, respectively (12). To date, six glycosyltransferases involved in CS biosynthesis have been identified (Fig. 1B): chondroitin sulfate synthase-1 (CSS-1)/chondroitin synthase (CSy) (13), chondroitin sulfate synthase-2 (CSS-2)/chondroitin polymerizing factor (ChPF) (14, 15), chondroitin sulfate synthase-3 (CSS-3) (16), chondroitin sulfate glucuronyltransferase (CSGlcAT) (17), chondroitin sulfate N-acetylgalactosaminyltransferase-1 (18, 19), and 2 (20, 21) (CSGalNAcT-1, 2). All these enzymes have an N-terminal transmembrane domain and are localized to the Golgi apparatus where CS biosynthesis takes place (22). CSS-1, CSS-2, CSS-3, and CSGlcAT form a family of glycosyltransferase enzymes. CSS-1, CSS-2, and CSS-3 contain two glycosyltransferase domains and exhibit both N-acetylgalactosaminyltransferase (GalNAcT) and glucuronyltransferase (GlcAT) activities in chain elongation. Thus, they have glycosyltransferase-II (both GalNAcT-II and GlcAT-II) activity. CSGlcAT has an inactive GalNAcT domain and exhibits only GlcAT-II activity. CSGalNAcT-1 and 2 have one glycosyltransferase domain and exhibit GalNAcT activity in both the initiation and elongation processes, indicating that CSGalNAcT-1 and 2 have both GalNAcT-I and II activities. Thus, CS chain elongation may involve all six enzymes, whereas CS chain initiation likely involves only CSGalNAcT-1 and 2. Although individual enzymes have been biochemically characterized, the in vivo mechanism of CS biosynthesis is not fully understood. Since cartilage contains a large amount of CS, chondrocytes must be capable of efficiently synthesizing numerous CS chains. Elucidation of the mechanism of CS biosynthesis in cartilage would provide a basis for the development of a treatment promoting cartilage regeneration.

In this study, we identified CSGalNAcT-1 as the enzyme critical for cartilage CS biosynthesis. Overexpression of the enzyme in chondrocytic cells elevated the level of CS biosynthesis, and in vivo adenoviral gene delivery of the enzyme into the intervertebral disc resulted in a higher level of CS incorporation. The aggrecan synthesized in the CSGalNAcT-1-overexpressing cells had a higher number of CS chains. We term this molecule “super-aggrecan” and propose a novel enzyme-based approach for the therapeutic intervention of cartilage degenerative diseases.

Experimental Procedures

In situ hybridization — cDNA fragments encoding glycosyltransferases and aggrecan core protein were prepared by PCR using cDNA of differentiating ATDC5 cells and appropriate primers (Supplemental table 1). The PCR product was then subcloned into the pBluescript IISK(−) vector (Stratagene). RNA probes were prepared using cDNA prepared as described above and a DIG RNA labeling kit (Roche). A humerus was obtained from an E16.5 mouse and fixed with 4% paraformaldehyde for 16 h at 4 °C. The samples were embedded in paraffin and sliced into 4-μm sections. In situ hybridization was performed as described previously (23).

Cell culture — ATDC5 cells, a cell line established from chondrocytic cells of a mouse embryonal carcinoma, undergo all the steps of chondrogenesis (24). The cells were cultured in a maintenance medium consisting of a 1:1 mixture of Dulbecco’s modified Eagle medium (DMEM) and Ham’s F12 medium containing 5% fetal bovine serum (FBS), 10 µg/ml human transferrin, 3 × 10⁻⁸ M sodium selenite, penicillin, and streptomycin. To induce chondrocyte differentiation, the cells at confluency were treated with 10 µg/ml of bovine insulin. N1511 cells, a cell line established from chondrocytic cells from the rib cage of a 4-week-old male p53-null mouse, undergo all the steps of chondrogenesis (25). The cells were cultured in minimum essential medium alpha (α-MEM) supplemented with 10% FBS, penicillin, and streptomycin. Differentiation was similarly induced by combined treatment with of 1 × 10⁻⁶ M dexamethasone and 1 × 10⁻⁷ M rat parathormone. LTC cells, a rat chondrosarcoma cell line, were cultured in Ham’s F12 medium containing 10% FBS, penicillin, and streptomycin. 293A cells, an embryonic human kidney cell line, were cultured in DMEM containing 10% FBS, 0.1
mM MEM non-essential amino acids, 2 mM L-glutamine, penicillin, and streptomycin. Cell culture was performed at 37 °C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every other day.

Real time RT-PCR — mRNA was isolated from ATDC5, N1511, and transfected LTC cells and cartilage of cmd mouse at E18.5 using Micro-FastTrack™ (Invitrogen) according to the manufacturer’s instructions. cDNA was synthesized using Super Script™ First-Strand (Invitrogen) according to the manufacturer’s instructions. The TaqMan probes contained a reporter dye at the 5’ end and a quencher dye at the 3’ end. To calculate the copy number, control vectors were prepared by PCR using appropriate primers and the cDNA obtained from N1511 cells, followed by subcloning of the fragment into pCRII-TOPO (Invitrogen). For CSS-3, a BAC clone was used as the PCR template. Primers and probes used for real time RT-PCR are listed in Supplemental table 2. Primers for the control vector are listed in Supplemental table 3. Relative quantification of gene expression was performed using the Applied Biosystems ABI Prism 7700 sequence detection system (TaqMan). PCR reactions for all the samples were performed in triplicate in 96-well optical plates using 5 ng cDNA, 25 μl TaqMan Universal PCR Mastermix (Applied Biosystems), 100 nM probe, 100 nM of each primer, and water to a final volume of 50 μl.

Thermocycling conditions comprised an initial holding step at 50 °C for 2 min, 95 °C for 10 min and 50 cycles of 95 °C for 15 s and 60 °C for 60 s. To standardize mRNA levels, the primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal controls.

Construction of mammalian expression vectors of glycosyltransferases and establishment of their overexpressing cell lines — Full length human cDNA encoding the glycosyltransferases was amplified by PCR using the Marathon Ready™ cDNA obtained from human bone marrow tissue (Clontech) as the template and two primers: 5’-AACGTTCCTCCCAAGCTTATGCATCGCC GCGGGGCTG-3’ and 5’-TCTAGACGGGATCCCTAGTGTTGC CCTGCTCC-3’ for CSGalNAcT-1; 5’-AACGTTCCTCCAAAGCTTACCATGCGAC CCCAGCTC-3’ and 5’-TCTAGAGCTCTAAGCTTATGCGAC CGCGGC-3’ for CSS-2; 5’-AAGCTTCCCAAGCTTTACGGCATTGCC CGCGGGGCTG-3’ and 5’-TCTAGAGCTCTAGACTAAGCTTATGG CCTGCTCC-3’ for CSS-3; 5’-AACGTTCCTCCCAAGCTTACCATGCGAC CCCAGCTC-3’ and 5’-TCTAGAGCTCTAAGCTTATGCGAC CGCGGC-3’. Relative quantification of these enzymes were measured by real time RT-PCR. The stable cells overexpressing individual enzymes at similar levels were used.

Alcian blue staining — Cells were plated in 6-multiwell plates. When the cells reached 80% confluency in a conditioned medium on day 2, they were washed in PBS and fixed with 4% paraformaldehyde for 30 min. A humerus was obtained from an E16.5 mouse and fixed with 4% paraformaldehyde for 16 h at 4 °C. The samples were embedded in paraffin and sliced into 4-μm sections. Transduced vertebral sections were obtained as described below. The cells and deparaffinized sections were then stained using 0.1% alcian blue in 0.1 M HCl at room temperature for 10 min.

Immunostaining — The transfected LTC cells were plated in 4-well culture slides. When the cells reached 30% confluency at day 2, they were washed in PBS and fixed with 4% paraformaldehyde for 30 min. They were double-immunostained as previously described (25). For chondroitin 4-sulfate (C4S) detection, mouse monoclonal anti-C4S (LY111, 1/200, Seikagaku) and Alexa Fluor™ 488-conjugated anti-mouse IgM (1/1000) were used as the primary and secondary antibodies, respectively. For
Characterization of aggrecan — The transfected LTC cells were plated in a 100-mm culture dish. The cells and conditioned medium were collected separately at day 2 and extracted with 4 M guanidine hydrochloride (GuHCl), 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 mM N-ethylmaleimide (NEM). The samples were stirred overnight at 4 °C and clarified by centrifugation in capped polycarbonate tubes (15,000 × g, 10 min, 4 °C). Portions of the samples were used for immuno-dot blot analysis. Cesium chloride (CsCl) was added to obtain a density of 1.55 g/ml, this was followed by ultracentrifugation under a dissociative condition in polyallomer tubes at 40,000 × g at 10 °C for 70 h. This ultracentrifugation at a slightly higher density enabled separation of the various aggrecan populations. The solution in the tube was fractionated into nine tubes—D1–D9—from the bottom. Aggrecan separation was monitored by immuno-dot blot analysis.

Immuno-dot blot analysis — The samples were applied to nylon N+ membranes by dot blot using the BIO-DOT™ Apparatus (BIO-RAD). The membranes were immunodetected as previously described with slight modification (4). They were pretreated with 1 unit/ml protease-free chondroitinase ABC (Seikagaku). For aggrecan core protein detection, rabbit polyclonal anti-aggrecan (×1000) (26) and Alexa Fluor™ 594-conjugated anti-rabbit IgG (×1000) were used as the primary and secondary antibodies, respectively. The specimen was observed using a Zeiss LSM 5 Pascal laser confocal microscope.

Characterization of CS chains — The transfected LTC cells were plated in a 100-mm culture dish. After 2 days, they were labeled with [35S] sulfate (100 µCi/ml) for 24 h. The cells and conditioned medium were collected separately at day 3 and extracted with 0.2 M NaOH for 16 h at room temperature. Next, they were neutralized by the addition of 4 M acetate and digested with 1mg/ml proteinase K in 50 mM Tris-HCl, pH 8.0 for 2 h at 37 °C. The samples were applied to a DEAE-Sephardel (Amersham Bioscience) column that was equilibrated with 50 mM Tris-HCl, pH 7.5. After washing with 10 column volumes of 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, glycosaminoglycan-rich fractions were eluted with 50 mM Tris-HCl, pH 7.5, 2 M NaCl. The eluates were precipitated by the addition of 3 volumes of 95% ethanol containing 1.3% potassium acetate, and the precipitate was dissolved in 1 ml distilled water. A portion (100 µl) of the sample was further isolated by treatment with a mixture of 10 µU/ml heparitinase I (Seikagaku), 5 µU/ml heparitinase II (Seikagaku), and 10 µU/ml heparinase (Seikagaku) in 20 mM acetate buffer, pH 7.0, 2 mM calcium acetate for 2 h at 37 °C. Separation was performed using an Ultrafree-MC filter cup (Millipore), and dissolved in 200 µl distilled water. To evaluate [35S] sulfate incorporation, a portion (100 µl) of the sample was measured by scintillation-counting. To examine the chain lengths, the other portion (100 µl) of the sample was applied to a Superose 6 column (Amersham Bioscience) equilibrated in 50 mM Tris-HCl, pH 7.5, and 0.2 M NaCl. Elution of the CS chains was monitored by scintillation counting. In separate experiments, heparitinase-resistant glycosaminoglycan fractions were prepared form the conditioned medium of non-labeled LTC cells as described above. The sample was labeled with [3H]-labeled sodium borohydride as described previously (27). After removal of free [3H]-labeled sodium borohydride using a Sephadex G-25 spin column, the labeled sample (~90,000 cpm) was applied to a Superose 6 column under the same condition as above.

Analysis of CS disaccharide composition — Transfected LTC cells were plated in a 100-mm culture dish. The cells and conditioned medium were collected separately at day 2. Glycosaminoglycans were isolated by β-elimination, protease digestion, and DEAE-Sephacel column chromatography, as described above. The eluates were precipitated by the addition of 3 volumes of 95% ethanol containing 1.3% potassium acetate, and the precipitate was dissolved in 100 µl distilled water. The samples were treated with 30 mU chondroitinase ABC in 25 µl 50 mM Tris-HCl, pH 7.5, 0.04% BSA for 2 h at 37 °C, and filtered using Ultrafree-MC (5,000 molecular weight limit). Unsaturated disaccharides in the filtrates
were analyzed by reverse phase ion-pair chromatography using the Senshu Pak column Docosil with a fluorescence detector according to Toyoda’s method (28) with a slight modification of elution conditions. The modified gradient program was as follows: 0-10 min, 1-4% eluent B; 10-11 min, 4-10% eluent B; 11-20 min, 10-18% eluent B; 20-22 min, 18-70% eluent B; 22-29 min, 70% eluent B.

Construction of the CSGalNAcT-1 adenovirus expression vector, viral particle production, and in vivo transduction to vertebral disc — cDNA encoding human CSGalNAcT-1 was prepared by PCR using the primers (5′-CACCAGTATGGTTCGGC-3′, 5′-TGTGGTTTTTCATTGTTCTG-3′) and CSGalNAcT-1/pcDNA3.1 (+) as the template. This cDNA was then subcloned into pAd/CMV/V5-DEST (Invitrogen) using pENT/D-TOPO (Invitrogen). Adenoviral particles were obtained by transfecting 293A cells with the CSGalNAcT-1 adenoviral expression plasmid, and the titer was checked according to the manufacturer’s instructions (ViraPower™ Adenoviral Expression System, Invitrogen). Adenoviral particles prepared using Adenoviral Packaging System (Invitrogen) were used as the negative control. The particles (1.5 × 10⁷ plaque-forming units) were injected into the caudal intervertebral discs of 4-month-old ICR mice. One week after the injection, the mice were sacrificed, and the caudal vertebrae were immersed in 4% paraformaldehyde at 4 °C overnight and decalcified with K-CX solution (Fujisawa) for 24 h at room temperature. The samples were then embedded in paraffin and sliced into 4-μm sections.

RESULTS

Expression patterns of glycosyltransferases involved in CS biosynthesis in cartilage — The growth plate cartilage contains chondrocytes at different stages of differentiation; resting, proliferative, prehypertrophic, and hypertrophic chondrocytes (29). During differentiation, chondrocytes at the prehypertrophic stage show the highest level of aggrecan synthesis (30). Since the CS chains are attached to the core protein of aggrecan in cartilage, the expression of the key enzymes required for CS biosynthesis is likely upregulated, correlating with the expression of aggrecan core protein during chondrocyte differentiation. Initially, we investigated the expression of glycosyltransferases involved in CS biosynthesis in mouse developing cartilage. By in situ hybridization, CSS-1, CSS-2, CSGlcAT, and CSGalNAcT-1 were shown to be expressed in the prehypertrophic zone of the growth plate at E16.5, colocalized with aggrecan core protein. In particular, the expression of CSGalNAcT-1 was expressed at the highest level. In contrast, CSS-3 and CSGalNAcT-2 were expressed at very low levels (Fig. 2).

Next, we examined the expression patterns of the CS biosynthetic enzymes in ATDC5 cells, which reflect the in vivo chondrocyte differentiation process (24, 31). The expression of both CSGlcAT and CSGalNAcT-1 was increased similar to that of aggrecan core protein, whereas response was less pronounced for that of CSS-1, CSS-2, and CSGalNAcT-2. The expression of CSS-3 was not detected (Fig. 3). Another chondrogenic cell line N1511 (25) displayed expression patterns similar to those of CS biosynthetic enzymes during differentiation (data not shown). These in vitro cell culture results indicate that the expression of CSGlcAT and CSGalNAcT-1 correlates well with that of aggrecan core protein.

Real time RT-PCR showed a high level of CSGalNAcT-1 expression and lower levels of CSS-1, CSS-2, and CSGlcAT expression in cartilage at E18.5 (Fig. 4B, open bar); this is consistent with the in situ hybridization data. We further investigated the correlation between CS biosynthetic enzymes and aggrecan expression, using cartilage isolated from mice with cartilage matrix deficiency (cmd), known as natural aggrecan-null mice (32, 33). As the heterozygote and homozygote cmd cartilage exhibited ~50% and ~9% aggrecan gene transcription, respectively (Fig. 4A), quantification of the enzyme expression in the WT, heterozygote, and homozygote cartilage could facilitate the identification of enzymes critical for CS biosynthesis. In the cmd heterozygote cartilage, CSGalNAcT-1 expression was diminished to ~30% that of the WT, whereas expression of the other enzymes was constantly low (Fig. 4B, gray bar). In the cmd homozygote cartilage, CSGalNAcT-1 expression further decreased to a low level similar to that of the other
enzymes (Fig. 4B, black bar). These results, demonstrating high levels of gene expression in cartilage and a good correlation with aggrecan core protein expression, suggested that CSGalNAcT-1 mainly regulates CS synthesis in cartilage.

**CSGalNAcT-1 overexpression enhances CS biosynthesis in chondrocytic cells** — Next, we examined whether CSGalNAcT-1 overexpression could enhance CS biosynthesis in LTC cells. LTC cells are derived from a rat chondrosarcoma and have the characteristics of mature chondrocytes, including a high level of aggrecan expression (34, 35). Cells stably transfected with the CSGalNAcT-1 expression plasmid overexpressed human CSGalNAcT-1 mRNA by approximately 80-fold compared with endogenous rat mRNA expression (data not shown). Metabolic labeling with \[^{35}S\] sulfate showed up to a ~2.2-fold increase in CS levels in both the conditioned medium and the cell lysates of the CSGalNAcT-1-overexpressing cells compared with the mock-transfected cells. In contrast, stable clones overexpressing comparable levels of CSS-1, CSS-2, and CSGlcAT did not show increased CS biosynthesis (Fig. 5A). A stronger staining intensity with alcian blue confirmed the increased CS biosynthesis in the CSGalNAcT-1-overexpressing cells (Fig. 5B). These results indicate that while the activity of CSS-1, CSS-2, and CSGlcAT in LTC cells is saturated, that of CSGalNAcT-1 is not and that CSGalNAcT-1 overexpression further enhances CS biosynthesis.

**Aggrecan in CSGalNAcT-1-overexpressing cells contains a larger number of CS chains** — Aggrecan core protein expression may have been upregulated by CSGalNAcT-1 overexpression, as shown by the correlation between the expression in ATDC5 and N1511 cells and that in cm cartilage. However, real time RT-PCR revealed similar mRNA levels of aggrecan core protein in both CSGalNAcT-1 and mock-transfected LTC cells (Fig. 6A). Dot blot analysis showed similar levels of aggrecan core protein in the conditioned media and cell lysates of both transfected cell types (Fig.6B). Since these data suggested that aggrecan synthesized in the CSGalNAcT-1-overexpression cells contains a larger CS amount per molecule, we examined the density of aggrecan. Cesium chloride (CsCl) density gradient ultracentrifugation followed by dot blot detection of aggrecan core protein showed that aggrecan obtained from the CSGalNAcT-1-overexpressing cells was present in the bottom fractions, whereas aggrecan from the mock-transfected cells was widely distributed (Fig. 6C). Immunohistochemically, CS stained stronger in the pericellular zone of the CSGalNAcT-1-overexpressing cells than in the mock-transfected cells, whereas aggrecan core protein stained at similar levels (Fig. 6D). These observations indicate that aggrecan in the CSGalNAcT-1-overexpressing cells contains a larger amount of CS.

The increased amounts of CS imply that there is an increased number of CS chains or greater elongation of individual CS chains in GSGalNAcT-1-transfected cells. To examine CS chain length, we performed metabolic labeling of the CS chains synthesized in the transfected cells and subjected them to gel chromatography. The elution profile demonstrated CS chains with peaks at the same position (Fig. 7A, arrows). Furthermore, direct molecular sieve analysis of \[^{3}H\]-labeled CS chains confirmed the same size of CS chains (Fig. 7B). These results indicate that the CS chain length in GSGalNAcT-1 and mock-transfected cells was similar, although there was an ~2.2-fold increase in CS incorporation in the CSGalNAcT-1-overexpressing cells. The functions of the CS chains such as water absorption, depend on their saccharide structure including sulfation (36). When the CS disaccharide composition was analyzed, a similar ratio of hexuronic acid (HexA)-GalNAc, HexA-GalNac(4S), and the other disaccharides was observed in both the GSGalNAcT-1- and mock-transfected cells (9.9%, 88.2%, and 1.9% for mock; 4.3%, 93.6%, and 2.1% for CSGalNAcT-1-overexpressing cells; Fig. 7C). Therefore, CSGalNAcT-1 overexpression appeared to cause a 2.2-fold increase in the number of CS chains along with a similar sulfation level per chain.

**In vivo gene delivery of CSGalNAcT-1 increases CS biosynthesis in cartilage** — Our in vitro studies using cell lines demonstrated that CSGalNAcT-1 overexpression increases CS biosynthesis. Since chondrocytes in vivo may utilize a similar mechanism, we tested whether in vivo gene delivery of CSGalNAcT-1 increases CS...
biosynthesis in cartilage. A replication-deficient adenovirus 5 containing a cDNA encoding CSGalNAcT-1 and the V5 epiteope tag was injected into the nucleus pulposus of the intervertebral discs of 4-month old mice, and its expression was confirmed by immunostaining for the V5 tag (data not shown). Histological analysis at day 7 after the injection showed intense alcian blue staining in the pericellular zone of the nucleus pulposus cells in the disc and chondrocytes in the vertebral endplate compared to mice injected with the control adenovirus (Fig. 8). These results demonstrated that in vivo gene delivery of CSGalNAcT-1 in fact increased CS biosynthesis in cartilage.

DISCUSSION

In this study, we identified CSGalNAcT-1 as a key enzyme for CS biosynthesis in cartilage. Analysis of the expression patterns of glycosyltransferases and aggrecan core protein in normal developing cartilage by in situ hybridization, in chondrogenic cell lines, and in the aggrecan-null cartilage of cmd mice demonstrated both high-level expression of CSGalNAcT-1 and good correlation of its expression with that of aggrecan core protein. Furthermore, CSGalNAcT-1 overexpression in cultured chondrocytic cells and the intervertebral disc elevated CS synthesis in the cells. These observations suggest that CSGalNAcT-1 is still unsaturated in chondrocytes and an increase in the level of CS via its overexpression leads to prevention of cartilage destruction.

Out of the six glycosyltransferases involved in CS biosynthesis, CSGalNAcT-1 expression was the highest in E18.5 mouse cartilage exhibiting ~170-fold higher expression than that of CSGalNAcT-2 (Fig. 4B). In tissues other than cartilage, CSGalNAcT-1 is expressed at the highest level in the thyroid gland and placenta, which exhibit expression only up to 1.5–5-fold that of CSGalNAcT-2 (20). In addition, various cell lines show relatively low levels of CSGalNAcT-1 expression compared with other enzymes ((18) and Sakai, unpublished data). Thus, the high expression of CSGalNAcT-1 appears to be characteristic of chondrocytes and cartilage. CSGalNAcT-1 expression correlated well with that of aggrecan core protein that provides acceptor sites for the glycosaminoglycan chains. CSGalNAcT-1 expression was closely associated with chondrocyte differentiation. As CSGalNAcT-1 expression was diminished accordingly in the heterozygote and homozygote cmd cartilage, the expression of aggrecan core protein may regulate that of CSGalNAcT-1. Aggrecan core protein as substrate may be required for enzyme complex and its decrease may deliver a feedback signal toward downregulation of CSGalNAcT-1 expression. Alternatively, aggrecan in the cartilage matrix may maintain the chondrocyte phenotype including high level expression of CSGalNAcT-1. Thus far, we have not identified cis-elements that are specific to chondrocyte differentiation in the promoter region of the CSGalNAcT-1 gene. The transcriptional regulation of this gene remains to be studied.

We demonstrated the presence of aggrecan with a larger number of attached CS chains in CSGalNAcT-1-overexpressing cells (Fig. 9). We designate this molecule “super-aggrecan” as it would more efficiently contribute to cartilage function. Since CSGalNAcT-1 has stronger initiating activity than elongating activity (18), chain initiation may be the rate-limiting step in CS biosynthesis and chondrocytes may have sufficient machinery for CS chain elongation following initiation. Indeed, overexpression of CSS-1, CSS-2, and CSGlcAT did not increase CS biosynthesis. The other enzyme involved in chain initiation, CSGalNAcT-2, showed consistent low expression in cartilage and has lower initiation activity than CSGalNAcT-1 (20), excluding any major involvement in cartilage CS biosynthesis. Enhanced CS biosynthesis by CSGalNAcT-1 overexpression suggests the presence of a large number of linkage regions as acceptor substrates in the native aggrecan. Although the aggrecan core protein has more than a hundred putative Ser-Gly sequences of the CS-attachment sites (2), approximately a half number of them may be attached only with the linkage tetrasaccharides. However, stubs of the linkage region have not been identified in aggrecan, although such stubs are present in a part-time CS proteoglycan thrombomodulin, an integral membrane glycoprotein expressed on endothelial cell surfaces (37). Recently, glucuronyltransferase-I (GlcAT-I), which transfers a GlcA residue to the second Gal residue in the linkage region, has been
shown to increase CS biosynthesis to ~1.5-fold (38). Thus, an immature linkage region may be present, and the enzyme complex that includes GlcAT-I and CSGalNAcT-1 may catalyze the completion of the linkage region and CS chain initiation.

Lines of evidence that support CSGalNAcT-1 as the critical enzyme for CS biosynthesis in various tissues as well as cartilage have been presented. Syndecan-4 in CSGalNAcT-1-overexpressing COS7 cells contains a large amount of CS (20). CSGalNAcT-1 overexpression in Balb/3T3 cells, a cell line established from normal mouse fibroblast cells, achieves ~5-fold CS synthesis (Sakai, personal data). Taken together, these observations strongly suggest that chain initiation by CSGalNAcT-1 is a critical step in the regulation of CS biosynthesis and overexpression of the enzyme would substantially increase CS synthesis in various tissues.

To date, many approaches to promote cartilage regeneration have been attempted, including the treatment of mesenchymal stem cells with growth factors to promote their differentiation into chondrocytes and the implantation of chondrocytes incorporated into scaffolds that maintain cellular phenotype and support the tissue structure (39-42). These interventions are based on the notion that aggrecan is synthesized only by chondrocytes and that it is essential for cartilage function. However, because of the difficulty in maintaining aggrecan synthesis in chondrocytes, regeneration of functional cartilage has not yet been achieved. In contrast to the above approaches, our research focused on CS as the critical factor that determines cartilage function and successfully generated “super-aggrecan.” As the turnover of aggrecan in cartilage is slow, with a half-life of approximately ten years (43-45), the function of “super-aggrecan” will most probably be maintained.

Adenoviral gene delivery of CSGalNAcT-1 enhanced in vivo CS synthesis in nucleus pulposus cells and endplate chondrocytes. In contrast, we have been unsuccessful in gene delivery to the articular cartilage (data not shown), although a recent similar study demonstrated increased CS expression in an articular cartilage explant (38). Since CS biosynthesis occurs in the Golgi apparatus (22), addition of the enzyme to the tissue is ineffective and overexpression in the cell is essential for this enzyme-based increase in CS levels. Effective gene delivery of CSGalNAcT-1 would be necessary to evaluate the prevention of cartilage destruction.

In conclusion, this study identified the glycosyltransferase critical for CS biosynthesis and proposes a novel strategy for the treatment of cartilage degenerative disorders, distinct from cell-based approaches that focus on chondrocyte differentiation and aggrecan core protein expression. The affects of “super-aggrecan on chondrocyte homeostasis and its susceptibility to aggrecan-degrading proteinases remain to be performed.
Sci. licr SGlcAT) exhibits chloride; HexA, hexuronic acid; and ser, J.K., Risbud, M., Wuisman, P., Meisel, H.J., Tannoury, C., cT ural comparison of the six glycosyltransferases. Conserved domains in hybridization patterns of the glycosyltransferases involved in CS biosynthesis and eresis. CS biosynthesis is initiated  by the elongation (glycosyltransferase N glycosyltransferases involved in chondroitin sulfate synthase have been identified. Chondroitin sulfate synthase elongation (glycosyltransferase N glycosyltransferases involved in chondroitin sulfate synthase have been identified. Chondroitin sulfate synthase elongation (glycosyltransferase N glycosyltransferases involved in chondroitin sulfate synthase have been identified. Chondroitin sulfate synthase elongation (glycosyltransferase N glycosyltransferases involved in chondroitin sulfate synthase have been identified. Chondroitin sulfate synthase elongation (glycosyltransferase N glycosyltransferases involved in chondroitin sulfate synthase have been identified. Chondroitin sulfate synthase elongation (glycosyltransferase N glycosyltransferases involved in chondroitin sulfate synthase have been identified. Chondroitin sulfate synthase elongation (glycosyltransferase N glycosyltransferases involved in chondroitin sulfate synthase have been identified. Chondroitin sulfate synthase elongation (glycosyltransferase N glycosyltransferases involved in chondroitin sulfate synthase have been identified. Chondroitin sulfate synthase elongation (glycosyltransferase N glycosyltransferases involved in chondroitin sulfate synthase have been identified. Chondroitin sulfate synthase elongation (glycosyltransferase N glycosyltransferases involved in chondroitin sulfate synthase have been identified. Chondroitin sulfate synthase elongation (glycosyltransferase N glycosyltransferases involved in chondroitin sulfate synthase have been identified. Chondroitin sulfate synthase C4S, chondroitin 4 sulfate. The abbreviations used are: CS, chondroitin sulfate; GalNAc, N-acetylgalactosamine; GlcA, glucuronic acid; Gal, galactose; CSS-1, chondroitin sulfate synthase-1; CSS-2, chondroitin sulfate synthase-2; CSS-3, chondroitin sulfate synthase-3; CGlAT, chondroitin sulfate glucuronyltransferase; CGlNAcT-1, chondroitin sulfate N-acetylgalactosaminyltransferase-1; CGlNAcT-2, chondroitin sulfate N-acetylgalactosaminyltransferase-2; GalNAcT, N-acetylgalactosaminyltransferase; GlcAT, glucuronyltransferase; cmd, cartilage matrix deficiency; CsCl, Cesium chloride; HexA, hexuronic acid; C4S, chondroitin 4-sulfate.

FOOTNOTES
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The abbreviations used are: CS, chondroitin sulfate; GalNAc, N-acetylgalactosamine; GlcA, glucuronic acid; Gal, galactose; CSS-1, chondroitin sulfate synthase-1; CSS-2, chondroitin sulfate synthase-2; CSS-3, chondroitin sulfate synthase-3; CGlAT, chondroitin sulfate glucuronyltransferase; CGlNAcT-1, chondroitin sulfate N-acetylgalactosaminyltransferase-1; CGlNAcT-2, chondroitin sulfate N-acetylgalactosaminyltransferase-2; GalNAcT, N-acetylgalactosaminyltransferase; GlcAT, glucuronyltransferase; cmd, cartilage matrix deficiency; CsCl, Cesium chloride; HexA, hexuronic acid; C4S, chondroitin 4-sulfate.

FIGURE LEGENDS

Fig. 1. CS biosynthesis and its related glycosyltransferases. (A) A schematic diagram of the catalytic activities of the glycosyltransferases involved in chondroitin sulfate synthesis. CS biosynthesis is initiated by the transfer of an N-acetylgalactosamine (GalNAc) unit to the linkage region of a GlcA-Gal-Gal-Xyl tetrasaccharide primer that is attached to a serine residue of the core protein. This is followed by chain elongation that occurs by the alternate addition of GalNAc and glucuronic acid (GlcA) residues. Six glycosyltransferases involved in CS synthesis have been identified. Chondroitin sulfate synthase-1 (CSS-1), chondroitin sulfate synthase-2 (CSS-2), and chondroitin sulfate synthase-3 (CSS-3) exhibit both N-acetylgalactosaminyltransferase (GalNAcT) and glucuronyltransferase (GlcAT) activities in chain elongation (glycosyltransferase-II activity). Chondroitin sulfate glucuronyltransferase (CGlAT) exhibits only GlcAT-II activity. Chondroitin sulfate N-acetylgalactosaminyltransferase-1 and 2 (CGlNAcT-1, 2) exhibit GalNAcT activity in both the initiation and elongation processes (GalNAcT-I and II activities). (B) A schematic structural comparison of the six glycosyltransferases. Conserved domains in β3GalTs or β4GalTs are indicated as closed or open boxes. The putative transmembrane (TM) domains are indicated as gray boxes.

Fig. 2. In situ hybridization patterns of the glycosyltransferases involved in CS biosynthesis and aggrecan core protein in developing cartilage. The expression patterns of aggrecan core protein (Aggrecan), CSS-1, CSS-2, CSS-3, CGlAT, and CGlNAcT-1 and CGlNAcT-2, in a mouse humerus at E16.5 are shown together with the staining pattern with alcian blue. Note that CSS-1, CSS-2,
CSGlcAT, and CSGalNAcT-1 are expressed in the prehypertrophic zone of the growth plate at E16.5 and colocalized with aggrecan core protein. CSGalNAcT-1, in particular, showed the highest level of expression.

Fig. 3. Expression patterns of the glycosyltransferases involved in CS biosynthesis during chondrocyte differentiation of the ATDC5 cells. The expression levels were standardized with those of GAPDH and plotted as a fold increase against the level at confluency (day 0). Note the correlated expression of CSGlceAT and CSGalNAcT-1 with that of aggrecan core protein.

Fig. 4. The endogenous mRNA levels of aggrecan core protein and glycosyltransferases in wild type, heterozygote, and homozygote cmd cartilage. (A) mRNA levels of the aggrecan core protein are shown as a % of WT E18.5 cartilage. (B) mRNA levels of glycosyltransferases are shown as the copy number (+/+, wild type; cmd/+, heterozygote cmd; cmd/cmd, homozygote cmd). Note the high expression of CSGalNAcT-1 and its good correlation with that of aggrecan core protein.

Fig. 5. Increased CS levels following the overexpression of CSGalNAcT-1 in LTC cells. Stable transfectants overexpressing CSGalNAcT-1, CSS-1, CSS-2, CSGlceAT, and mock transfectants were established by transfecting LTC cells with expression vectors of the enzyme genes followed by selection with G418 for 10 days. (A) [35S] sulfate incorporation of mock-transfected cells, CSGalNAcT-1, CSS-1, CSS-2, and CSGlceAT-overexpressing cells are shown (gray bar, cell lysates; black bar, conditioned medium). Note that CSGalNAcT-1-overexpressing cells exhibit an ~2.2-fold increase in CS biosynthesis compared with the mock-transfected cells, while the others do not. (B) Culture plates stained with alcian blue (pH 1.0). CSGalNAcT-1-overexpressing cells exhibited a stronger staining intensity than the mock-transfected cells.

Fig. 6. Characterization of aggrecan in CSGalNAcT-1-overexpressing cells. The aggrecan in CSGalNAcT-1-overexpressing cells contains a large amount of CS. (A) mRNA levels of aggrecan core protein measured by real time RT-PCR. (B) Immunoblot of aggrecan core protein in cell lysates and conditioned medium. (C) Immunoblot analysis of aggrecan core protein after CsCl density gradient ultracentrifugation. Note that aggrecan in CSGalNAcT-1-overexpressing cells is mainly present in the bottom fractions (fraction 1 and 2), whereas that in mock-transfected cells is widely distributed in fractions 1–6. The density (g/ml) of each fraction is 1.638 for D1, 1.615 for D2, 1.600 for D3, 1.573 for D4, 1.525 for D5, 1.512 for D6, 1.465 for D7, 1.460 for D8, and 1.420 for D9, respectively. (D) Immunostaining for aggrecan core protein and chondroitin-4 sulfate (C4S). C4S stains stronger in the pericellular zone of CSGalNAcT-1-overexpressing cells than in the mock-transfected cells, whereas the aggrecan core protein stained at similar levels.

Fig. 7. Analysis of CS chains in CSGalNAcT-1-overexpressing cells. (A) Elution profiles of Superose 6 gel chromatography. The cells were metabolically labeled with [35S] sulfate for 24 h. Glycosaminoglycans, extracted as indicated in Methods, were applied to the Superose 6 column. The samples from CSGalNAcT-1 overexpressing cells (closed circle) and the mock-transfected cells (open circle) are shown. Note that two peaks are located at the same position (arrows). (B) Elution profiles of Superose 6 gel chromatography. CS-rich fractions prepared from the conditioned medium were labeled with [3H]-labeled sodium borohydride, and were applied to Superose 6 column. The samples from CSGalNAcT-1 overexpressing cells (bold line) and the mock-transfected cells (narrow line) are shown. Note that two peaks are at the same position. Vt indicates the position of the total volume. (C) Disaccharide composition of CS in CSGalNAcT-1 and mock transfectants. HexA-GalNAc (open bar), HexA-GalNAc(4S) (gray bar), and others (black bar) are shown as a % of the saccharide content. Hexuronic acid (HexA)-GalNAc, HexA-GalNAc(4S), and the other disaccharides were observed to have similar ratios in both GSGalNAcT-1- and mock-transfected cells.

Fig. 8. Increased CS biosynthesis by in vivo gene delivery of CSGalNAcT-1. Histological sections of mouse intervertebral discs at 4 months of age, which were stained with alcian blue (pH 1.0), 7 days after an injection of adenoviral particles. Low magnification of the intervertebral disc (A, B), higher
magnification of nucleus pulposus (C, D) and the endplate (E, F) injected with mock (A, C, E) and CSGalNAcT-1 (B, D, F) adenovirus are shown. Note the intense alcian blue staining in the pericellular zone of the nucleus pulposus cells in the disc and chondrocytes in the vertebral endplate of mice with CSGalNAcT-1 gene delivery compared to mice injected with the control adenovirus.

Fig. 9. **A schematic diagram of aggrecan synthesized by control and CSGalNAcT-1-overexpressing chondrocytes.** (A) Native aggrecan, (B) aggrecan in CSGalNAcT-1-overexpressing cells, which contains ~2.2-fold the number of CS chains attached to the core protein; designated “super-aggrecan” (blue, CS chain). G1, G1 globular domain; G2, G2 globular domain; G3, G3 globular domain; and KS, keratan sulfate.
**A**

Chondroitin sulfate

GsINAcT-1,2  GSICAT  GSINAcT-1,2

CSS-1,2,3  linkage region

Initiation activity (glycosyltransferase-I)

Elongation activity (glycosyltransferase-II)

GlcA
GalNAc
Galactose
Xylose

Core protein

**B**

|   | |3 GT domain (GlcAT activity) | |4 GT domain (GalNAcT activity) |
|---|---|-----------------------------|-----------------------------|
| CSS-1 |   |                             |                             |
| CSS-2 |   |                             |                             |
| CSS-3 |   |                             |                             |
| GSICAT |   |                             |                             |
| GSINAcT-1 |   |                             |                             |
| GSINAcT-2 |   |                             |                             |

**Fig. 1**
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 9
Chondroitin sulfate N-acetylgalactosaminyltransferase-1 plays a critical role in chondroitin sulfate synthesis in cartilage

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