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〈**Regular Article**〉

Chondroitin Sulfate Disaccharide Enhances Extracellular Matrix-Related Gene and Protein Expression in Normal Human Dermal Fibroblasts *in Vitro*

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Abstract

The physiological effects of chondroitin sulfate (CS) depend on the extent of its sulfation. This study focused on the effects of highly sulfated CS with different molecular weights in normal human dermal fibroblasts (NHDFs). NHDFs were treated with each CS. The expression of various genes was assessed by real-time PCR. Type I collagen content and elastin protein expression levels were assessed by ELISA and Western blotting, respectively. Disaccharide CS significantly increased the expression of genes required for extracellular matrix (collagen, type I, alpha I, decorin, elastin, lysyl oxidase, SMAD2 and SMAD3). In addition, we confirmed that collagen and elastin increased at the protein level. In contrast, high- and low-molecular-weight CS polymer had no significant effect. Therefore, highly sulfated disaccharide CS increased the expression of extracellular matrix-related genes and proteins. It may be possible that the highly sulfated disaccharide CS may increase firmness and elasticity of skin.

Key words: disaccharide chondroitin sulfate, type I collagen, decorin, elastin, lysyl oxidase.

1. Introduction

Chondroitin sulfate (CS) is a physiologically active substance that retains moisture and is present in various parts of the body. It is a water-soluble polymer, a linear chain formed by linking disaccharides of *N*-acetylgalactosamine and glucuronic acid. CS is present in the form of proteoglycans, which attach to core proteins *in vivo*. CS proteoglycans include decorin (DCN), versican and aglycan.

CS is a glycosaminoglycan, important in extracellular matrix-related proteins in the body. For example, it has been reported that CS increases the expression of elastin (ELN) in smooth muscle cells[1)] and that DCN (CS proteoglycan) is required for stabilizing of collagen fibers[2)]. This suggests that CS is an important factor in fibril formation.

Collagen fibers and elastic fibers are the main fibers in the skin, and type I collagen is the main component of collagen fiber in skin. As DCN is required to stabilize collagen fibers[2)], type I collagen and DCN are important for collagen fiber formation in the skin. ELN is the main component of elastic fibers. ELN is a key extracellular matrix protein that is critical for elasticity and resilience. SMAD2 and SMAD3 are phosphorylated in response to TGF-β, inducing SMAD complex formation. The complex translocates into the nucleus to activate the transcription of ELN genes[3)]. Therefore, ELN, SMAD2 and SMAD3 are important for elastic fiber formation. Lysyl oxidase (LOX) plays an important role in cross-linking elastic fibers and collagen fibers[4)].

This study investigated the effects of CS on the extracellular matrix-related genes collagen, type I, alpha I (*COL1A1*), *DCN*, *ELN*, *LOX*, *SMAD2* and *SMAD3*. CS is present in the skin, and has a low degree of sulfation, affecting the viability of fibroblasts[5)–8)]. However, the effects of CS vary depending on the degree of sulfation. For example, CS with a high degree of sulfation promotes neurite outgrowth[9)] and suppression of pits formed by osteoclasts, but these effects were not observed with CS having low sulfation[10)]. In addition, the interaction between CS and proteins varies depending on the extent of sulfation[11)–15)]. Therefore, degree of sulfation is an important factor for effectiveness evaluation of CS, but there is little information on the effects of highly sulfated CS.

In contrast, hyaluronan is a member of the glycosaminoglycans, which includes the CS; its physiological effects differ depending on its molecular weight. It is therefore conceivable that the effects of CS are also dependent on molecular weight.

We aimed to elucidate physiological function of highly sulfated CS with different molecular weights. In this paper, we examine the effects of molecular weight of CS with 3 sulfate groups per unit (high degree of sulfation) by investigating the effects on gene expression of *COL1A1*, *DCN*, *ELN*, *LOX*, *SMAD2* and *SMAD3*, and protein expression of type I collagen and ELN, which are important for fibril formation in normal human dermal fibroblasts.

2. Materials and Methods

2–1. Materials

High-molecular-weight CS (average M.W.: 47,000 Da, sulfate group content: 38.57%) and low-molecular-weight CS (average M.W.: 3,700 Da, sulfate group content: 38.74%) (high sulfation) were obtained from Maruho Co., Ltd. (Osaka,

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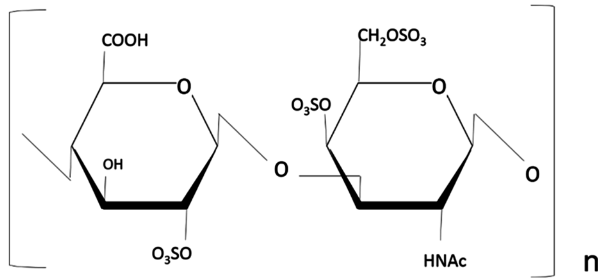

Fig. 1. Structure of CS. Disaccharide CS: $n=1$, low molecular weight CS: $n=4$, high molecular weight CS: $n=60$–70.

Japan). Fig. 1 shows the structure of highly sulfated CS. Disaccharide CS (ΔUA-2S→GalNAc-4S-6S Na_2, M.W.: 707 Da) was purchased from Dextra Laboratories, Ltd. (Earley Gate, Reading, UK). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3,3′,5,5′-Tetramethylbenzidine (TMB) solution was obtained from R&D Systems, Inc. (McKinley Place, MN, USA). Other reagents and chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2–2. Cell culture

Normal human dermal fibroblasts (NHDFs) were purchased from Kurabo Industries, Ltd. (Osaka, Japan). NHDFs were cultured in DMEM with 10% FBS, under 5% CO_2, at 37℃.

2–3. Cell viability

The effects of each CS treatment on cell viability were measured by MTT assay. NHDFs were seeded at 0.5×10^4 cells/well in a 24 well plate (AGC Techno Glass Co., Ltd., Funabashi, Chiba, Japan), treated with CS and cultured for 24 h. An MTT stock solution (5 mg/m*l*) was mixed with medium (ratio=1 : 9). Medium containing MTT solution was added to each well and was incubated for 2 h with 5% CO_2 at 37℃. The formazan crystals that developed in each well were dissolved in 0.04 mol HCl/isopropyl alcohol, and were measured using a scanning multi-well spectrophotometer (570 nm).

2–4. RNA preparation and real-time polymerase chain reaction (RT-PCR)

NHDFs were seeded at 5.0×10^4 cells/well in a 60-mm dish (AGC Techno Glass Co., Ltd.), treated with CS in DMEM in 10% FBS and cultured for 12 h. Total RNA was extracted from cultured NHDFs treated CS using RNAiso Plus (TaKaRa Bio Inc., Otsu, Shiga, Japan). The quantity and quality of extracted total RNA was measured by determining the 260/280 nm ratio. RNA was synthesized to cDNA by reverse-transcription using a Prime Script™ reagent kit (Perfect Real Time) (TaKaRa Bio Inc.) and a Veriti 96-well thermal cycler (Life Technologies Corporation, Foster City, CA, USA). Real-time PCR was performed using SYBR Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa Bio Inc.) and a Step One Plus ™ Real Time PCR system (Life Technologies Corporation). Table 1 shows the primers used.

Table 1. Primers used for real-time PCR.

Primer	Forward Primer	Reverse Primer
COL1A1	5′-CCGCCGCTTCACCTACAGC-3′	5′-TTTTGTATTCAATCACTGTCTTGCC-3′
DCN	5′-TGTCATAGAACTGGGCACCAAT-3′	5′-GGAAAGCCCCATTTTCAATTC-3′
ELN	5′-GGGCAATTCCTGGAATTGGA-3′	5′-CTGCTTCTGGTGACACAACCC-3′
LOX	5′-TTGTGCGCTGTGACATTCG-3′	5′-TTCCCACTTCAGAACACCAGG-3′
Smad2	5′-ACTAACTTCCCAGCAGGAAT-3′	5′-GTTGGTCACTTGTTTCTCCA-3′
Smad3	5′-CTGTGTGAGTTCGCCTTCAA-3′	5′-AATGGCTGTAGTCGTCCAGT-3′
*GAPDH**	5′-GAAGGTGAAGGTCGGAGT-3′	5′-GAAGATGGTGATGGGATTTC-3′

**GAPDH*: glyceraldehyde 3-phosphate dehydrogenase

2–5. Quantitative determination of type I collagen (competitive enzyme-linked immuno sorbent assay: ELISA)

ELISA for quantitative determination of type I collagen was carried out according to the method of Rennard *et al*[15]. Briefly, NHDFs were seeded at 8.0×10^4 cells/well in a 96-well plate (AGC Techno Glass Co., Ltd.), treated with CS in DMEM in 1% FBS and cultured for 72 h. NHDFs were dissolved to extract protein (2% sodium dodecyl sulfate (SDS), 10% glycerol, 6.27 mM Tris–HCl, pH 6.8), and culture medium was collected. Extracted cell protein concentration was determined using Lowry's method. Proteins (human type I collagen; Daiichi Fine Chemical, Takaoka, Toyama, Japan) were coated on an ELISA plate (AGC Techno Glass Co., Ltd.) at 1.25 μg/m*l* overnight at room temperature. Type I collagen-coated plates were washed with PBS-T (PBS in 0.05% Tween 20), and blocked using blocking solution (BlockAce; DS Pharma Biomedical, Tokyo, Japan) at 37℃ for 1 h. The culture medium were mixed with primary antibody (Biotin-anti-type I collagen antibody; Rockland, Limerick, PA, USA), and were incubated at 37℃ for 1 h. Culture medium mixed with the primary antibody was added to the wells of the protein-coated plate, followed by incubation at 37℃ for 1 h. Protein-coated plates were then treated with Streptavidin-horseradish peroxidase (R&D Systems, Inc., Minneapolis, MN, USA), and incubated at 37℃ for 1 h. TMB solution was added to protein-coated plates, followed by incubation at room temperature. Subsequently, stop solution was added, and measurements were obtained with a scanning multi-well spectrophotometer (450 nm). A standard curve was generated using human type I collagen (Daiichi Fine Chemical) (0, 0.078, 0.158, 0.313 and 0.625 μg/m*l*).

2–6. ELN determination (Western blotting)

NHDFs were seeded at 5.0×10^4 cells/well in a 60-mm dish (AGC Techno Glass Co., Ltd.), treated with CS in DMEM in 10% FBS and cultured for 48 h. Proteins were extracted from NHDFs in the presence of disaccharide CS using a protein extraction reagent (2% SDS, 10% glycerol, 6.27 mM Tris–HCl, pH 6.8). Concentration of the extracted proteins was determined using the Lowry assay. Extracted proteins were mixed 1 : 1 with SDS-PAGE sample buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 1% bromophenol blue), and were heated at 95℃ for 3 min. Proteins were separated by SDS-PAGE using 7% or 10% acrylamide gels, and were transferred to a PVDF membrane at 100 V for 30 min. PVDF membrane with transferred protein was blocked with blocking buffer (5% skim milk, 1% Tween 20, 0.13 M NaCl, 0.02 M Tris–HCl, pH

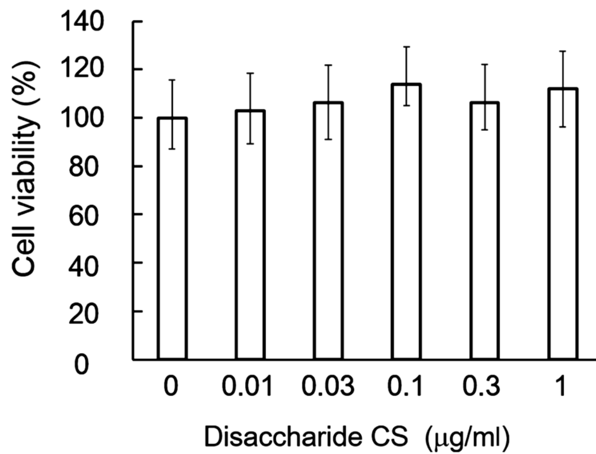

Fig. 2. Effects of various concentrations of disaccharide CS on cell viability in normal human dermal fibroblasts. NHDFs were treated for 24 h with disaccharide CS of different concentrations. Cell viability was determined by MTT assay. Values are means±*S.D.* ($n=4$).

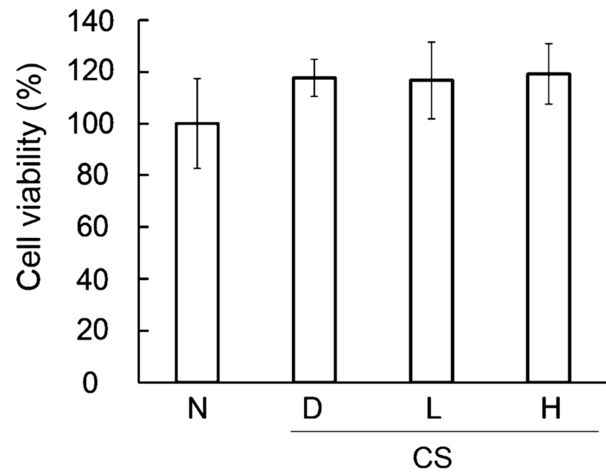

Fig. 3. Effects of various molecular weights of CS on cell proliferation of normal human dermal fibroblasts. NHDFs were treated for 24 h with CS (concentration: 0.3 g/m*l*). Cell proliferation was determined by MTT assay. Values are means±*S.D.* ($n=4$). D is disaccharide CS, L is low molecular weight CS and H is high molecular weight CS.

7.2). PVDF membrane with transferred protein was incubated with primary antibodies (Rabbit Anti-elastin polyclonal antibody, Unconjugated; Bioss, Woburn, MA, USA; *β*-actin (13E5) Rabbit mAb; Cell Signaling Technology, Inc., Danvers, MA, USA), washed with TBS-T (1% Tween 20, 0.13 M NaCl, 0.02 M Tris–HCl, pH 7.2), and incubated with secondary antibody (ECL™ Anti-Rabbit IgG, Horseradish Peroxidase linked whole antibody from Donkey; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham™ ECL™ Prime Western Blotting Detection Reagent; GE Healthcare). Intensity of chemiluminescence was detected using a LAS-1000plus (FUJIFILM Corporation, Tokyo, Japan) and was analyzed by Multi Gauge software (FUJIFILM Corporation).

2–7. Data analysis

Data represent means and standard deviation (n=3–6). Analysis was conducted using Tukey's multiple statistical test or Student's *t*-test with SAS.

3. Results

3–1. Cell viability and proliferation

NHDFs were treated with disaccharide CS and cell viability was determined after 24 h. There were no changes in viability at any concentration of disaccharide CS ($p>0.05$) (Fig. 2). In addition, there were no changes in cell viability on treatment with 0.3 *μ*g/m*l* disaccharide CS, low-molecular-weight CS or high-molecular-weight CS ($p>0.05$) (Fig. 3).

3–2. Gene expression levels

NHDFs were treated with CS of various molecular weights, and RNA was recovered after 12 h. Gene expression levels of *COL1A1*, *DCN*, *ELN*, *LOX*, *SMAD2* and *SMAD3* were then determined using real-time PCR. Disaccharide CS significantly increased gene expression levels of *COL1A1*, *DCN*, *ELN* and *SMAD2* when compared with the normal group and the high-molecular-weight CS-treated group ($p<0.05$). In particular, disaccharide CS significantly increased expression levels of *COL1A1* and *DCN* when compared with other groups ($p<0.05$). In addition, disaccharide CS tended to increase expression levels of *LOX* and *SMAD3* when compared with the normal group ($0.05<p<0.1$). In contrast, low-molecular-weight CS significantly increased expression levels of *SMAD2* when compared with the normal group ($p<0.05$), and tended to increase expression levels of *COL1A1* ($0.05<p<0.1$). However, high-molecular-weight CS did not affect gene expression levels ($p>0.1$) (Figs. 4a–f).

3–3. Type I collagen and ELN expression level

Type I collagen content was quantified using ELISA. Type I collagen content was significantly higher in the disaccharide CS-treated group ($p<0.05$).

ELN protein expression levels were determined by Western blot. Protein expression levels of ELN were significantly higher in the disaccharide CS-treated group ($p<0.05$).

4. Discussion

We focused on the effects of highly sulfated CS with different molecular weights in normal human dermal fibroblasts (NHDFs). First, the effects of CS treatment on cell viability were measured using the MTT assay. No cytotoxicity was observed at any concentration of disaccharide CS, or for low-molecular-weight or high-molecular-weight CS at 0.3 *μ*g/m*l*. NHDFs were treated with CS of different molecular weights in order to evaluate the gene expression levels of extracellular matrix-related proteins after 12 h. Disaccharide CS significantly increased gene expression levels of *COL1A1*, *DCN*, *ELN* and *SMAD2* when compared with the normal group, and tended to increase expression levels of *LOX* and *SMAD3* ($0.05<p<0.1$). However, high-molecular-weight CS did not have a significant effect on gene expression levels. Type I collagen, a protein required for the formation of collagen fibers, is abundant in the skin. DCN is required to stabilize collagen fibers.

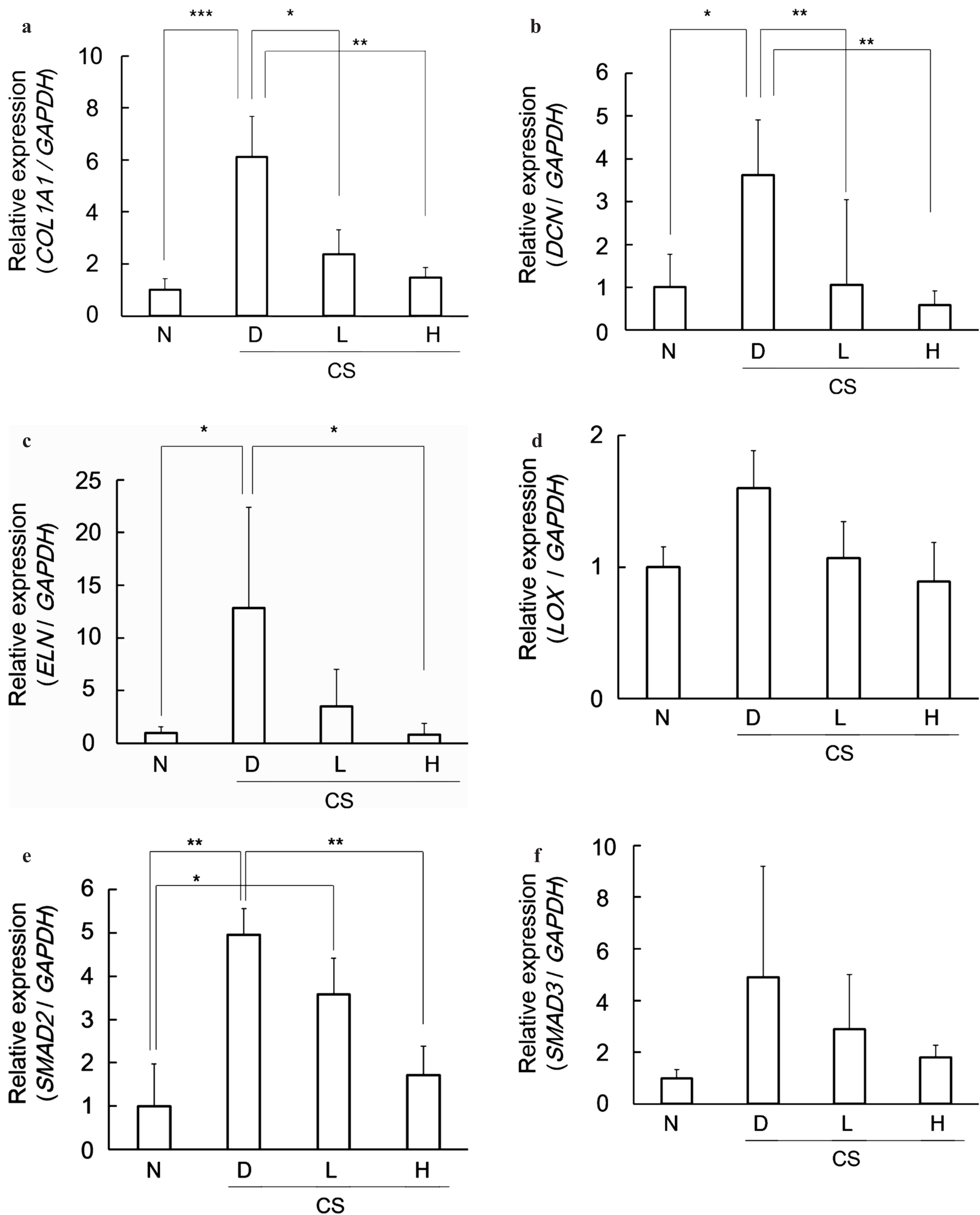

Fig. 4. Effects of various molecular weights of CS on mRNA expression levels of extracellular matrix-related proteins in NHDF. NHDFs cells were incubated with 0.1 μg/m*l* of various molecular weight of CS (or medium) and gene expression levels were determined for (a) *COL1A1*, (b) *DCN*, (c) *ELN*, (d) *LOX*, (e) *SMAD2* and (f) *SMAD3*. Gene expression was measured by real-time PCR after exposure to CS for 12 h. Results show relative quantification where gene expression in control samples is "1". mRNA levels were normalized by dividing by the quantity of *GAPDH* in each sample. Values are means±*S.D.* of at least three experiments (n = 3–4). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, as compared with untreated cells. Statistical analysis was conducted using Tukey's multiple statistical tests. D is disaccharide CS, L is low molecular weight CS and H is high molecular weight CS.

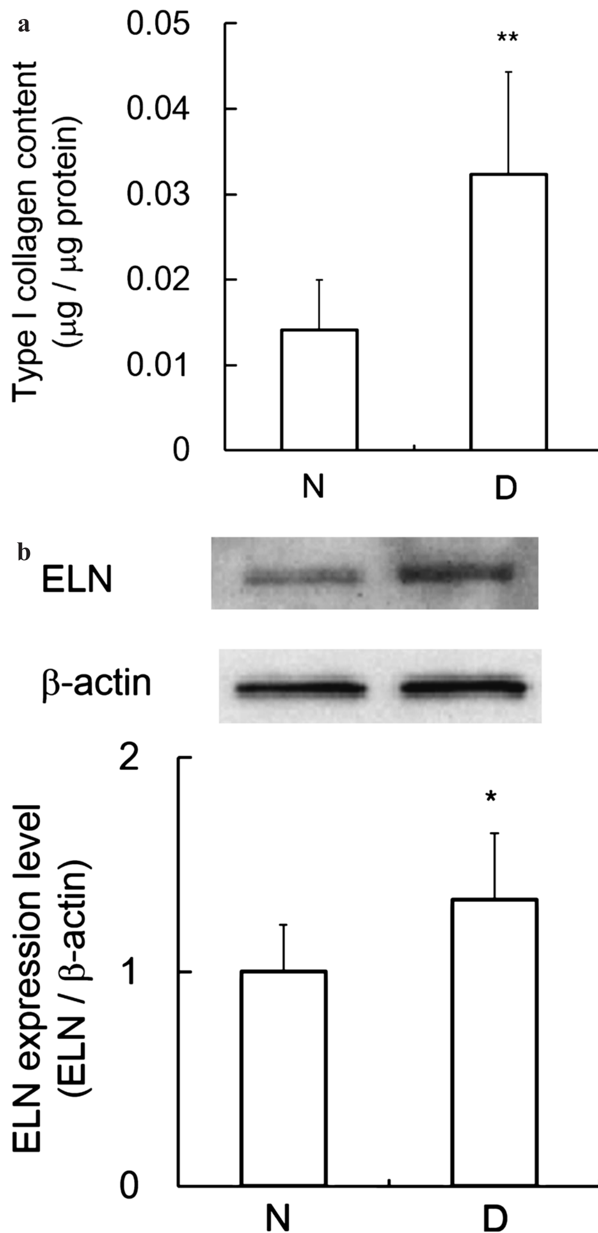

Fig. 5. Effects of disaccharide CS on type I collagen content and ELN proteins expression levels in NHDF. Type I collagen content and ELN proteins expression levels were determined by ELISA (a) and Western blotting (b), respectively. The band of ELN and *β*-actin were detected an apparent molecular mass of approximately 65 kDa and 45 kDa, respectively. Values are means± *S.D.* of at least three experiments ($n=3$−6). *: $p<0.05$, **: $p<0.01$, 360 when compared with untreated cells. Statistical analysis was conducted using Student's *t*-test.

ELN is the main component of elastic fibers in skin. LOX plays an important role in cross-linking fibers. SMAD2 and SMAD3 are necessary for the production of ELN. All six proteins are important for fibril formation in the skin. We investigated type I collagen content and ELN protein expression levels after treatment with disaccharide CS having a high degree of sulfation, and found that type I collagen content and ELN protein expression levels were significantly higher in the disaccharide CS-treated group. Thus, disaccharide CS with a high degree of sulfation can potentially act on fibroblasts to promote fiber formation.

Hyaluronan is a glycosaminoglycan whose effects vary depending on its molecular weight (e.g., high molecular weight or low molecular weight)[16–18]. High-molecular-weight hyaluronic acid and tetrasaccharide hyaluronan also have different effects on the *COL1A1* gene[19]. Here, we showed that CS with a high degree of sulfation has different effects depending on its molecular weight (Fig. 4). Heparin is a glycosaminoglycan. The interaction between heparin and IL-10 varies depending on the molecular weight[20]. Therefore, it is possible that the affinity of CS binding to protein (e.g., receptor, cytokine) depends on the molecular weight of CS.

CS increased *SMAD2* and *SMAD3* gene expression levels. SMAD2 and SMAD3 are proteins in the SMAD/TGF-*β* pathway. CS is known to bind to TGF-*β*[21]. Furthermore, TGF-*β* induced signaling events in human articular chondrocytes are influenced by exogenous chondroitin sulfate[22]. Therefore, highly sulfated disaccharide

CS might act via the SMAD/TGF-*β* pathway. However, the mechanisms for the expression of extracellular matrix-related proteins are unknown. Whether highly sulfated disaccharide CS binds directly to TGF-*β* or TGF receptor requires further investigation. However, it is also possible that CS has its own receptor. Whether the affinity of CS-binding receptors are molecular-weight dependent requires further investigation.

Highly sulfated disaccharide CS can be delivered into the skin because of its low molecular weight, and as disaccharide CS increased the expression of extracellular matrix-related genes necessary for fibril formation, it can be expected to improve the firmness and elasticity of skin.

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