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NOTE

Anatomy

Preliminary study of the gene expression of sulfation and degradation enzymes for chondroitin sulfate in glycerol-treated C2C12 myoblast cells

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ABSTRACT. In this study, we induced chemical damage of C2C12 myoblasts that had differentiated into myotubes with glycerol, and four sulfation enzymes for chondroitin sulfate (CS) [carbohydrate sulfotransferase (Chst) 12, Chst15 and Chst3 and uronyl 2-O-sulfotransferase (UST)] and two CS degradation enzymes [hyaluronidase (Hyal) 1 and Hyal2] were examined for changes in gene expression. Treatment of myoblasts with 5% glycerol significantly increased the expression levels of the sulfation enzymes *Chst12* and *Chst15* and the degradation enzymes *Hyal1 and Hyal2*. However, the expression levels of the other two genes (*Chst3* and *Ust*) showed no change. Differences in the expression levels of these enzymes may help to understand the difference in responsiveness of myoblasts to glycerol after muscle injury *in vivo* or *in vitro*.

KEY WORDS: cell culture, chondroitin sulfate sulfotransferase, glycerol, hyaluronidase, myoblast

Chondroitin sulfate (CS) is a type of glycosaminoglycan (GAG) that binds to core proteins and exists mainly in the extracellular matrix as a proteoglycan. The CS chain is a non-branched polysaccharide chain of variable length that consists of two alternating monosaccharides (repeating disaccharide structure) of D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc) [12, 15]. No sulfate groups or one or two sulfate groups are attached per disaccharide unit. Depending on the site and number of sulfate groups attached, CS is classified into CS-O, A, C, D, and E subtypes [16, 17]. The CS chain alone, that is, CS without sulfate group modification (CS-O), is converted to CS-A by chondroitin 4-O-sulfotransferase (C4ST; Chst12) and synthesized from CS-A to CS-E by GalNAc-4-Sulfate-6-O-sulfotransferase (GalNAc4S-6ST; Chst15) [6–8, 13]. On the other hand, CS-C is synthesized when CS-O is sulfated with chondroitin 6-O-sulfotransferase (C6ST; Chst3). Furthermore, when CS-C is sulfated by uronyl 2-O-sulfotransferase (UST), it becomes CS-D [5, 14]. Hyaluronidase (Hyal) is known as a CS degradation enzyme in addition to hyaluronan, and it has been reported that Hyal1 and Hyal2 are distributed in skeletal muscle tissue [1, 2, 11]. It has been reported that myodifferentiation is inhibited by CS, especially the oversulfated CS subtypes (CS-D and CS-E), which have two sulfate groups per disaccharide unit [16]. CS-treated myoblasts fused, but myotube formation (elongation) was inhibited, and the cells showed a similar circular shape [17]. On the other hand, reducing CS from a culture environment smoothly promotes myodifferentiation [11, 19]. Although CS is being recognized as a regulator of the processes of myodifferentiation and myoregeneration, there is little information on CS sulfation and degradation enzymes. Therefore, in this study, as a preliminary experiment to understand the myoregeneration and myodiffereniation *in vivo*, glycerol, which is one of the reagents that induces experimental damage to muscle tissue, was added to the culture medium of myoblasts to induce damage to the cells. Then changes in mRNA levels of CS sulfation enzymes and degradation enzymes in damaged myoblasts were analyzed.

In the experiments, mouse C2C12 myoblasts (RIKEN BioResource Center, Tsukuba, Japan) seeded in 12-well plates were used for cell morphological observation and genetic analysis, while cells in 96-well plates were used for cell counting. The amounts of medium (growth medium, differentiation medium, and glycerol-containing medium) per well used in the experiments were 1 ml and 0.1 ml for 12-well and 96-well plates, respectively. First, C2C12 cells were cultured in a growth medium (GM): Dulbecco's modified Eagle's medium (DMEM; GlutaMAX, Thermo Fisher Scientific, Tokyo, Japan) containing 10% fetal bovine serum (FBS, Fujifilm, Tokyo, Japan) and 1% antibiotics (penicillin-streptomycin solution ×100, Fujifilm). After becoming semi-confluent, the cells were seeded on collagen-coated plates (Collagen Type1-Coted Microplate 12 well, Iwaki, Tokyo, Japan) or 96-well plates

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(Tissue Culture Plate 96 wells, TPP, Trasadingen, Switzerland) at a density of 2×10^5 cells/ml. The seeded cells were cultured in a CO_2 incubator at 37°C and 5% CO_2 for 48 hr. Then, to induce myodifferentiation, the medium was replaced with DMEM medium containing 2% horse serum (Sigma-Aldrich Japan, Tokyo, Japan) and 1% antibiotics (differentiation medium: DM) and the cells were cultured in the DM for 6 days. During this period, the DM was changed at the third day after starting myodifferentiation. At the 6th day, the medium of each plate was changed to the GM and myodifferentiation was stopped. Next, glycerol adjusted with PBS to final concentrations of 5%, 10%, and 20% was mixed 1:1 with DMEM containing 10% FBS (glycerol medium), and the glycerol medium was added to the culture plates and incubated for 24 hr. As a control, a 1:1 mixture of PBS (without glycerol) and DMEM containing 10% FBS was used as a medium. For 96-well plates, cell viability was measured using a cell counting kit (CCK8, Dojin Kagaku Kenkyusho, Kumamoto, Japan) at the end of the culture period. The absorbance was measured at 450 nm using a microplate reader (Sunrise Remote, Tecan Group, Männedorf, Switzerland). Cells cultured on 12-well plates were observed with a microscope (IX71, Olympus, Tokyo Japan) and then lysed with TRIzol (Thermo Fisher Scientific) and collected. After synthesizing cDNA from the cells that had been collected, primers and RT-PCR reagents (Fast Stare Essential DNA Green Master, Roche, Basel, Switzerland) were added, and RT-PCR was performed on a real-time quantitative PCR system (Light Cycler Nano System, Roche). The PCR reaction consisted of 45 cycles of denaturation at 95°C for 5 sec, annealing at the annealing temperature [Only *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was reacted at 60°C and all others were reacted at 55°C.] of each primer for 4 sec, and extension at 72°C for 5 sec. The genes and primer sequences used in this analysis are summarized in Table 1. The expression level of each gene was determined using a calibration curve and calculated after normalization with *GAPDH*. Statistical analysis was performed using Stat View software version 5.0 (SAS Institute, Cary, NC, USA). Data on cell viability and *GAPDH* expression levels with glycerol treatment were analyzed by one-factor ANOVA and Bonferroni/Dunn's *post-hoc* test. The obtained data for expression of CS sulfation or degradation enzyme genes were evaluated by Student's *t*-test. $P<0.05$ was considered statistically significant.

Observation of the morphology of the cells after addition of glycerol showed that there was no significant change in cells with 5% glycerol compared to the control cells (no glycerol). However, gaps were observed between the cells with 10% glycerol, and the cells with 20% glycerol became rounder and smaller (Fig. 1A). Cell viability was significantly decreased ($P<0.01$) in cells treated with 10% and 20% glycerol compared to that of control cells (Fig. 1B). The expression level of the *GAPDH* gene was not significantly different in 5% glycerol-treated cells and control cells. However, the addition of 10% and 20% glycerol resulted in a significant decrease in the gene expression level of *GAPDH* in the cells compared to that in control cells ($P<0.01$) (Fig. 1C). Based on the low cell viability and the low expression level of *GAPDH*, we concluded that the 10% and 20% glycerol-treated cells were not suitable for analysis. In subsequent experiments, we decided to use only control cells and 5% glycerol-treated cells. The gene expression levels of *Chst12*, *Chst15*, *Hyal1*, and *Hyal2* were significantly increased by the addition of 5% glycerol compared to the

Table 1. Genes and primer sequences

Gene	Primer	Sequences (5′ to 3′)
GAPDH	Fw	AGGTCGGTGTGAACGGATTTG
	Re	TGTAGACCATGTAGTTGAGGTCA
Chst12	Fw	GCTGCAGAATCAGCATCACC
	Re	TCATGAGAGCCGACCCTAGT
Chst15	Fw	TATGACAACAGCACAGACGG
	Re	TGCAGATTTATTGGAACTTGCGAA
Chst3	Fw	GCTTTGCCTCAGGATTTCCG
	Re	ATAAGAGCAGGGCTGGGTCA
Ust	Fw	TCGTACCGTGGTCTTGCTTC
	Re	TAAATAGGGCTGTTCGGCGG
Hyal1	Fw	TGCCCGTAATGCCCTACGT
	Re	GCTGTGCTCCAGTTCCTCCA
Hyal2	Fw	CGAGGACTCACGGGACTGA
	Re	GGCACTCTCACCGATGGTAGA

Fw: forward, Re: reverse.

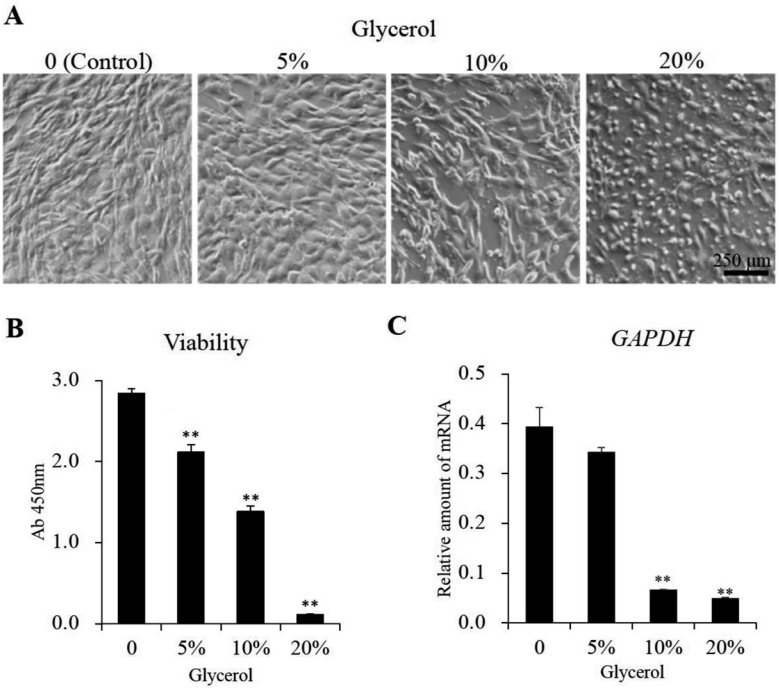

Fig. 1. (**A**) Morphology, (**B**) viability (n=4/group), and (**C**) expression level of *glyceraldehyde-3-phosphate dehydrogenase* (n=3/group) of C2C12 cells with addition of glycerol. Data in (B) and (C) are expressed as average ± standard deviation. **Indicates a significant difference between groups, $P<0.01$.

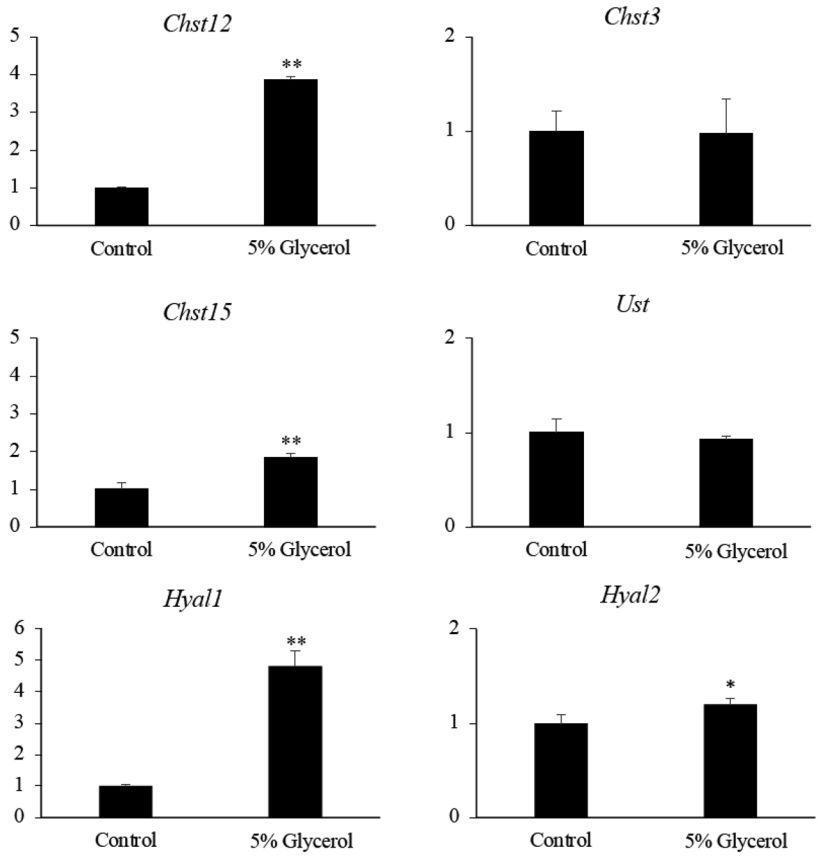

Fig. 2. Gene expression of chondroitin sulfate sulfation and degradation enzymes in response to the addition of 5% glycerol (n=3/group). The expression level of each gene is shown as relative ratio, with control as 1. Data are expressed as average ± standard deviation. * and ** indicate significant differences between groups, $P<0.05$ and <0.01, respectively.

levels in control cells ($P<0.05$). On the other hand, there was no difference in the gene expression level of *Chst3* or *Ust* with the addition of 5% glycerol (Fig. 2).

As mentioned above, there are two pathways for the sulfation of CS. One is CS-O to CS-A and then CS-A to CS-E (named "route A" for convenience), and Chst12 and Chst15 function as CS sulfation enzymes, respectively. The other sulfation route is believed to be from CS-O to CS-C and then from CS-C to CS-D (route B), and the sulfation enzymes of this route are Chst3 and UST. In this study, the expression of *Chst12* and *Chst15*, CS sulfation enzymes involved in route A was upregulated, while the expression of the enzyme genes involved in route B (*Chst3* and *Ust*) was not changed. These results suggest that glycerol has activity to upregulate the expression of genes (*Chst12* and *Chst15*) associated with CS sulfation of route A in myoblasts. Similarly, the expression levels of *Hyal1* and *Hyal2* also increased, but whether this is a "direct" response to glycerol or an "indirect" response to the increased synthesis of CS-A and CS-E, substrates of Hyal1 and Hyal2, by up-regulation of Chst12 and Chst15 has not been investigated. In our previous experiments, when glycerol was injected into muscle tissue of mice, the expression levels of the four CS sulfotransferases retrieved in the present study were all significantly increased on day 3 after glycerol injection; *Hyal1* and *Hyal2* were also increased by the same treatment (unpublished data). For example, CS-C, synthesized by Chst3, reduces the inflammatory response of macrophages by inhibiting nuclear translocation of nuclear factor-kappa B [18]. CS-C and CS-A have also been reported to inhibit the expression of pro-inflammatory cytokines and nitric oxide production in bone marrow-derived macrophages [3]. Furthermore, macrophages that migrate to inflammatory sites enhance the expression of some sulfotransferases [7, 9, 10], and platelets also secrete Hyal2 [4, 10]. Thus, CS and CS sulfation and degradation enzymes in inflammatory processes are likely to be closely related to the regulation of their expression and function. The fact that the expression levels of these two route B-related CS sulfation enzyme genes (*Chst3* and *Ust*) were not altered under culture conditions may be due to some inducible factors that are present *in vivo* but not in the culture environment. In the present study, we analyzed the expression of CS sulfation and degradation enzymes by glycerol treatment at the cellular level and found characteristic differences in the expression dynamics in C2C12 myoblasts. Differences in the expression of these enzymes may help to understand the difference in the responsiveness of myoblasts to glycerol after muscle injury *in vivo* or *in vitro*. Although the differences in the reactivity of these enzymes investigated in this study remain unclear and require further analysis, elucidation of the expression mechanisms of these CS sulfation and degradation enzymes will provide an important clue for a new enzyme-targeted strategy for smooth and rapid recovery of muscle tissue after injury.

CONFLICT OF INTEREST. All authors declare no conflict of interest associated with this manuscript.

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