

Research article

## HYDROGEN PEROXIDE AS A POTENTIAL MEDIATOR OF THE TRANSCRIPTIONAL REGULATION OF HEPARAN SULPHATE BIOSYNTHESIS IN KERATINOCYTES

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**Abstract:** Ionizing radiation is one of the types of oxidative stress that has a number of damaging effects on cutaneous tissues. One of the histological features of radiation-induced cutaneous fibrosis is the accumulation of extracellular matrix (ECM) components, including heparan sulfate proteoglycan (HSPG), which are required for the repair of tissue damage, and operate by interacting with a variety of growth factors. In this study, we established a model of human HaCaT keratinocytes overexpressing anti-oxidative enzyme genes to elucidate the mechanism of oxidative stress leading to the accumulation of HSPG and the role of its accumulation. Catalase overexpression induced an increase in anti-HS antibody (10E4) epitope expression in these cells. Western blotting showed that the smeared bands of HSPG were obviously shifted to a higher molecular weight in the catalase transfectants due to glycosylation. After heparitinase I treatment, the core proteins of HSPG were expressed in the

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Abbreviations used: CS – chondroitin sulfate; FGF – fibroblast growth factor; GAG – glycosaminoglycan; Gal – galactose; GalNAc – *N*-acetylgalactosamine; GlcA – glucuronic acid; GLCE – D-glucuronyl C5-epimerase; GlcNAc – *N*-acetylglucosamine; HSPG – heparan sulfate proteoglycan; HS2ST – heparan sulfate 2-*O*-sulfotransferase; HS6ST – heparan sulfate 6-*O*-sulfotransferase; PDGF – platelet-derived growth factor; ROS – reactive oxygen species; SOD – superoxide dismutase; XT – xylosyltransferase; Xyl – xylose

catalase transfectants to almost the same extent as in the control cells. In addition, the transcript levels of all the enzymes required for the synthesis of the heparan sulfate chain were estimated in the catalase transfectant clones. The levels of five enzyme transcripts – xylosyltransferase-II (XT-II), EXTL2, D-glucuronyl C5-epimerase (GLCE), HS2-O-sulfotransferase (HS2ST), and HS6-O-sulfotransferase (HS6ST) – were significantly increased in the transfectants. Moreover, hydrogen peroxide was found to down-regulate the levels of these enzymes. By contrast, siRNA-mediated repression of catalase decreased 10E4 epitope expression, the transcript level of HS2ST1, and the growth rate of HaCaT cells. These findings suggested that peroxide-mediated transcriptional regulation of HS metabolism-related genes modified the HS chains in the HaCaT keratinocytes.

**Key words:** Heparan sulfate proteoglycan, HaCaT keratinocyte, Glycosyltransferase, Catalase, Sulfotransferase

## INTRODUCTION

Heparan sulfate proteoglycan (HSPG) is one of the proteoglycans found on the cell surface and in the extracellular region. Its level increases in radiation-induced cutaneous lesions. Heparan sulfate (HS) chains can interact with a variety of growth factors that have heparin-binding domains, such as fibroblast growth factors (FGFs) and platelet-derived growth factor (PDGF) [1].

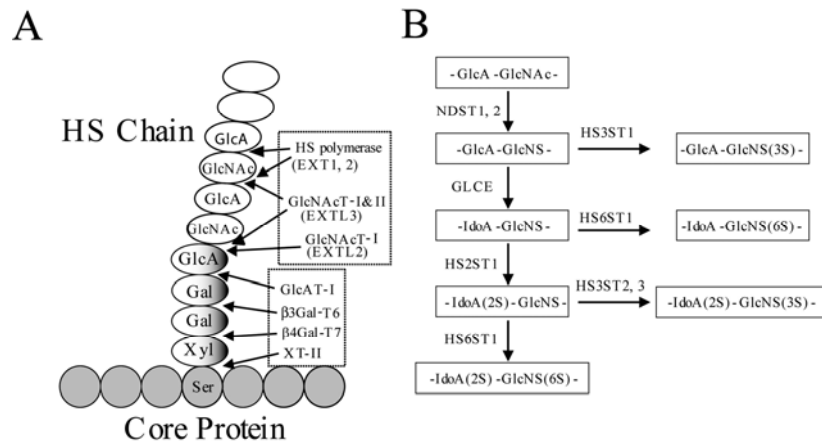


Fig. 1. The enzymes involved in the synthesis of the HS chain in HaCaT cells. 16 enzymes involved in synthesizing HS chains were expressed in HaCaT cells. A – The heparan sulfate (HS) chain has a backbone consisting of a repeat of alternating GlcA and GlcNAc on the common linkage tetrasaccharide, which is formed by the sequential transfer of monosaccharide residues at the reducing ends by each glycosyltransferase. B – The HS chain is subsequently modified by a C5-epimerase (GLCE) and various sulfotransferases: NDST-1, NDST-2, HS2ST1, HS6ST1, HS3ST1, HS3ST2, and HS3ST3.

The binding of growth factors to HSPG is essential for their local enrichment, enabling them to transfer to their high-affinity signaling receptors on the cell surface [2-4]. HSPG is composed of core proteins and covalently linked glycosaminoglycan (GAG) side chains, which have a backbone consisting of a repeat of alternating glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc) and the common linkage tetrasaccharide GlcA $\beta$ 1-3Gal $\beta$ 1-3Gal $\beta$ 1-4Xyl $\beta$ 1-O-Ser. Two xylosyltransferases (XT-I, -II) [5, 6], one xylosylprotein  $\beta$ 1,4-galactosyltransferase ( $\beta$ 4Gal-T7) [7], one  $\beta$ 1,3-galactosyltransferase ( $\beta$ 3Gal-T6) [8] and one glucuronyltransferase (GlcAT-I) [9] were cloned and identified as the glycosyltransferases involved in forming the linkage tetrasaccharide (Fig. 1). Five glycosyltransferases (EXT1, EXT2, EXTL1, EXTL2 and EXTL3) were cloned and shown to catalyze the polymerization of the HS chain [10]. In addition, it was found that five groups of enzymes, *N*-deacetylase/*N*-sulfotransferases (NDSTs), D-glucuronyl C5-epimerase (GLCE), heparan sulfate 2-*O*-sulfotransferase (HS2ST), heparan sulfate 6-*O*-sulfotransferases (HS6STs), and heparan sulfate 3-*O*-sulfotransferases (HS3STs), sequentially modify the backbone [11]. Each of the NDST [12-15], HS6ST [16], and HS3ST [17-19] enzyme families contains several members.

Exposing tissues to oxidative stresses induces the production of reactive oxygen species (ROS), which are important mediators of tissue damage [20]. Ionizing radiation is one of the types of oxidative stress that has a number of damaging effects on tissues through the mediation of reactive oxygen species (ROS). Additionally, ROS are known to be signaling molecules; hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) plays an especially important role in lymphocyte activation as a secondary messenger, mimicking the function of the ligand at the antigen receptor [21]. Several anti-oxidative enzymes work together to detoxify ROS in tissues. Superoxide dismutases (SODs) catalyze the disproportionation of superoxide radicals to molecular oxygen and hydrogen. They consist of two major types, the copper-and-zinc-containing type (Cu/ZnSOD) and the manganese-containing type (MnSOD). Catalase is another anti-oxidative enzyme distributed in the peroxisomes with the function of converting hydrogen peroxide to oxygen and water. It is able to modulate the level of a second messenger of metabolic oxidative stress via the scavenging of physiological hydrogen peroxide, so that it can both down-regulate growth signals and activate stress-associated signals [22]. However, the role of catalase in the pathogenesis of radiation-induced cutaneous fibrosis is not yet clear.

In this study, we investigated the effect of oxidative stress on the HSPG expression in a human keratinocyte cell line, HaCaT, using anti-oxidative enzyme gene transfectant cells as models. This study yielded the theory that oxidative stress regulates the formation of heparan sulfate chains through the transcriptional regulation of genes related to glycoconjugate metabolism.

## MATERIALS AND METHODS

### Cell culture and reagents

The human keratinocyte cell line HaCaT was provided by Dr. N. E. Fusenig (German Cancer Research Center) and maintained in a medium consisting of  $\alpha$ MEM (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. Anti-heparan sulfate (10E4) and anti-neo-heparan sulfate (3G10) monoclonal antibodies were obtained from Seikagaku Kogyo (Tokyo, Japan). Human recombinant FGF7 was purchased from R&D Systems.

### Transfection of the expression vectors

The coding sequences of catalase and MnSOD were amplified from cDNA derived from HaCaT cells by PCR. Each gene was amplified using a gene-specific primer set: catalase (sense: 5'-ggg gac aag ttt gta caa aaa agc agg ctc tat ggc tga cag ccg gga t-3', antisense: 5'-ggg gac cac ttt gta caa gaa agc tgg gtc tca cag att tgc ctt ctc c-3'); and MnSOD (sense: 5'-ggg gac aag ttt gta caa aaa agc agg ctg cat gtt gag ccg ggc agt g-3', anti-sense: 5'-ggg gac cac ttt gta caa gaa agc tgg gtc ata acg atc gtg gtt tac t-3'). Each PCR fragment was introduced into a pcDNA3.2-DEST expression vector according to the instruction manual of GATEWAY Cloning Technology (Invitrogen). The HaCaT cells were transfected with the expression vectors using FuGENE6 lipofection reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN), and selected in the presence of 1.5 mg/ml geneticin (G418; Life Technologies) for 2-4 weeks to obtain stable transfectants. Limiting-dilution cloning was performed to stabilize and enhance the proportion of transfectants with the desired phenotype.

### Transient siRNA assay

Stealth RNAi was obtained from Invitrogen. The synthesized oligonucleotides for the target site of each gene were: catalase, 5'-ggu aac cca gua gga gac aaa cuu a-3' and 5'-uaa guu ugu cuc cua cug ggu uac c-3'; and HS2ST1, 5'-agg aag agg aca ugg uga uca uuu a-3' and 5'-uaa aug auc acc aug ucc ucu ucc u-3'. Stealth RNAi against the *catalase* gene was conjugated with Alexa Fluor 546. The negative control Stealth RNAi was supplied by the manufacturer, and was transfected into HaCaT cells to obtain the corresponding negative control. The synthesized oligonucleotides for the negative control were 5'-ggu acc cga uga gag aaa cca auu a-3' and 5'-uaa uug guu ucu cuc auc ggg uac c-3'. Each Stealth RNAi was transfected at a final concentration of 50 nM using Lipofectamine RNAiMAX reagent according to the manufacturer's protocol (Invitrogen).

### Quantitative RT-PCR assay

A competitive RT-PCR assay was performed to quantify the transcripts of 24 genes related to glycoconjugate metabolism, catalase, MnSOD, and  $\beta$ -actin in HaCaT cells, as previously described [23, 24]. The DNA competitors were generated using the reagents of a Competitive DNA Construction kit, as

recommended by the manufacturer (Takara Bio Inc., Otsu, Japan). Competitive RT-PCR was performed using a Mastercycler (Eppendorf) in a total volume of 25  $\mu$ l of reaction buffer containing 5  $\mu$ l of standard plasmid DNA or sample cDNA, 5  $\mu$ l of competitor DNA at the optimal concentration, 0.2  $\mu$ M of each primer of the gene-specific primer set, and 0.5 U of ExTaq (Takara Bio Inc.). The PCR product was electrophoresed and analyzed with a DNA 1000 LabChip of a 2100 BioAnalyzer System (Agilent Technology Inc., Palo Alto, CA). The values for the transcripts were plotted on the respective standard curves to obtain the actual amount of each transcript. The level of GLCE transcripts was measured using a TaqMan probe, Hs01547874\_m1, using the 7500 Fast Real-Time PCR System (Applied Biosystems). The actual amount of each gene transcript (copies/ $\mu$ l) was divided by that of  $\beta$ -actin (copies/ $\mu$ l) for normalization (Tab. 1). Each normalized amount was further divided by the amount of the control sample to obtain the relative rate of expression (Figs 2, 4 and 5).

#### **Eliminative cleavage of HS chains**

HSPG was digested with heparitinase I (Seikagaku Kogyo, Tokyo, Japan) to remove the HS chains and expose the neo-heparan sulfate epitope. Cell pellets were lysed in an ice-cold solution containing 40 mM Tris-HCl (pH 8.0), 0.1% NP-40, 120 mM NaCl, and the protease inhibitors diethylenetriaminepentaacetic acid (DETAPAK) and protease inhibitor cocktail (Complete; Roche Applied Science). 100  $\mu$ g of protein were treated with 10 mU/ml heparitinase I (Seikagaku Kogyo, Tokyo, Japan) for 1 h at 37°C in a buffer containing 0.1 M sodium acetate and 2 mM calcium acetate (pH 7.0). The digested lysate was electrophoresed on an 8.5% SDS-polyacrylamide gel and subjected to western blot analysis.

#### **Western blot assay**

We estimated the amount of heparan sulfate and neo-heparan sulfate in each HaCaT transfectant by western blot analysis. The cell pellets were lysed in an ice-cold solution containing 40 mM Tris-HCl (pH 8.0), 0.1% NP-40, 120 mM NaCl, and the protease inhibitors diethylenetriaminepentaacetic acid (DETAPAK) and protease inhibitor cocktail (Complete; Roche Applied Science). 20 to 40  $\mu$ g of proteins separated by 8.5% SDS-PAGE were transferred to a nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Cell (Bio-Rad, Richmond, CA). The membrane was incubated with 1  $\mu$ g/ml of anti-heparan sulfate (10E4) or 1  $\mu$ g/ml of anti-neo-heparan sulfate (3G10) monoclonal antibody. The signal on the blot was visualized using Fuji X-ray film after treatment with ECL Plus Western Blotting Detection Reagents (GE Healthcare Bio-Sciences).

#### **Native gel assay**

Catalase activity was examined *in vitro* using non-denaturing polyacrylamide gel fractionation, as described previously [25]. Briefly, cell pellets were solubilized

by brief sonication in ice-cold potassium phosphate buffer (0.05 M, pH 7.8) with DETAPAK and Complete. 20  $\mu\text{g}$  of each sample of protein was fractionated by electrophoresis on a 7.5% native acrylamide gel. The gel was soaked in 50  $\mu\text{g}/\text{ml}$  horseradish peroxidase in phosphate buffer for 45 min, and then soaked for a further 10 min after the addition of hydrogen peroxide to a concentration of 5 mM. The gel was rapidly rinsed twice with distilled water, and then placed in 0.5 mg/ml diaminobenzidine in phosphate buffer until staining was complete [26].

#### **WST-1 assay**

Cell proliferation was assessed using the tetrazolium salt WST-1 according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Each type of cell was plated in 96-well plates at a density of  $5 \times 10^3$  cells per well, incubated for 24 h, and then transfected with Stealth RNAi as described above. FGF7 was added to serum-free  $\alpha\text{MEM}$  at a concentration of 1, 10 or 100 ng/ml 24 h before the WST-1 assay. After 24 h culture with FGF7, 10  $\mu\text{l}$  of WST-1 reagent was added to the 100  $\mu\text{l}$  culture medium in each well and then incubated for 4 h at 37°C. The absorbance at 450 nm was determined with an ELISA reader.

#### **Flow cytometry**

The HaCaT cells were subjected to flow cytometry to estimate the level of cell surface expression of HSPG. They were harvested in trypsin-EDTA, washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin, and incubated with anti-heparan sulfate antibody (10E4) for 30 min. Then they were washed twice and stained with the secondary antibody, FITC-conjugated-anti-mouse-IgM. A suspension of stained cells was subjected to FACSCalibur flow cytometry (BD Biosciences, CA) with fluorescence intensity measurement.

## **RESULTS**

#### **The increased expression of the anti-heparan sulfate antibody epitope (10E4) in catalase transfectants**

HaCaT cells were transfected with an expression vector containing the coding sequence of catalase or MnSOD in order to investigate the roles of anti-oxidative enzymes in the expression of the HS chain. Competitive RT-PCR analysis showed that the catalase transfectant (HaCaT-Catalase) had almost a 7-fold higher level of catalase transcripts than the control cells (HaCaT-pcDNA3) (Fig. 2A). Native gel assays indicated that the catalase transfectant cells clearly had higher catalase activities than the control cells (Fig. 2B). FACS analysis showed that the catalase transfectant cells were stained more brightly with the anti-HS antibody epitope (10E4) than the other transfectants. By contrast, the MnSOD transfectant cells were stained with anti-HS antibody at the same level as the control cells (Fig. 2C). This indicates that the expression of the anti-HS antibody epitope (10E4) in HaCaT cells occurred through a reduction in the level of hydrogen peroxide.

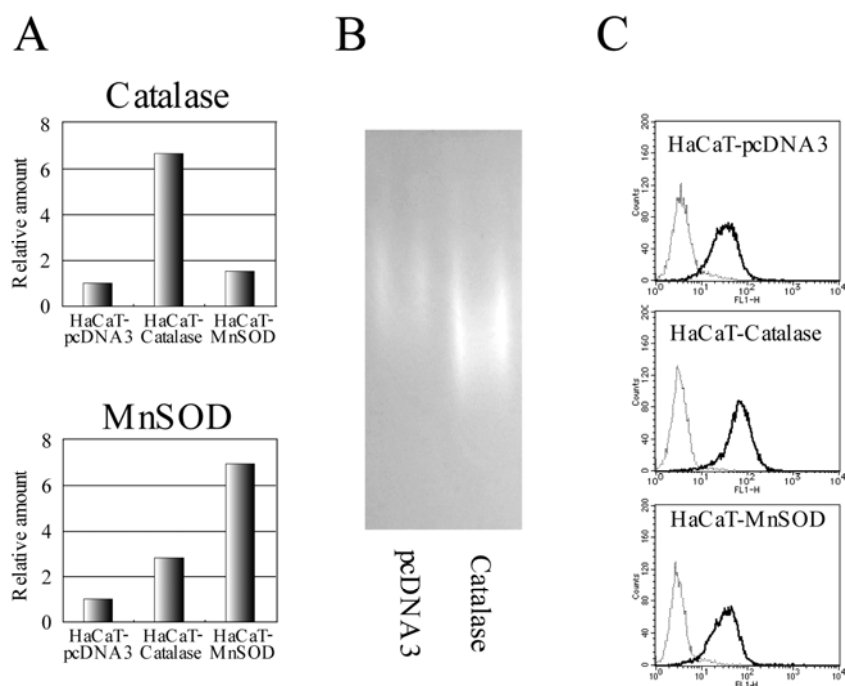


Fig. 2. The increased expression of the anti-heparan sulfate antibody (10E4) epitope in HaCaT cells transfected with anti-oxidative enzyme genes. HaCaT cells were transfected with the control vector (HaCaT-pcDNA3), catalase (HaCaT-Catalase), or MnSOD (HaCaT-MnSOD). A – The level of each transcript was measured by competitive RT-PCR. The absolute amount of each transcript (copies/ $\mu$ l) was normalized to the amount of  $\beta$ -actin, and in addition, each normalized value was further calculated as the value relative to that in HaCaT-pcDNA3 cells. B – The activity of catalase in the transfectants was measured in native gel assays. It was visualized as the density of a band. C – The transfectant cells were harvested from the dishes and subjected to flow cytometric analysis after staining with anti-heparan sulfate antibody (10E4).

#### The increased glycosylation of HSPG in the catalase transfectants

The cell lysate was subjected to western blot analysis using the anti-HS monoclonal antibody (10E4) to examine the expression level of heparan sulfate (HS) chains in the catalase transfectant cells (Fig. 3A). The catalase transfectants showed broad smears of high molecular weight molecules ranging in size from 70 to 200 kDa. By contrast, the mock transfectants showed broad smears which were mainly less than 150 kDa in size. The amount of high molecular weight smears was much higher in the catalase transfectants than in the mock transfectants. The mock transfectants also showed smears of smaller molecules ranging from 50 to 70 kDa; such smears were very faint in the catalase transfectants. After the eliminative cleavage of HS chains from HSPG with heparitinase I, single bands were detected at the same signal intensity in the

catalase and mock transfectants using anti- $\Delta$ HS monoclonal antibody (3G10; Fig. 3B). Anti- $\Delta$ HS monoclonal antibody reacts with the neo-epitope of heparan sulfate formed by the digestion of the intact epitope with heparitinase. The presence of these bands at the same signal intensity suggested that each type of transfectant cell expressed almost the same amount of core proteins of HSPG. The overexpression of catalase augmented the expression of heparan sulfate chains in the HaCaT cells, but did not affect the core protein synthesis.

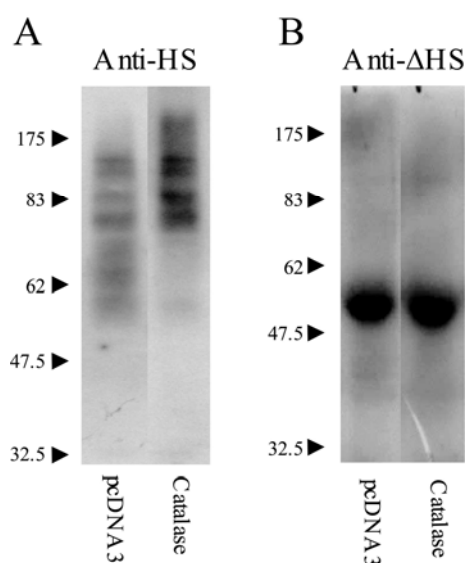


Fig. 3. The synthesis of heparan sulfate chains in the catalase transfectants. HaCaT cells were transfected with the control vector pcDNA3.2-DEST (pcDNA3) or the catalase expression vector (Catalase). A – Each lysate was electrophoresed on an 8.5% SDS-polyacrylamide gel and subjected to western blot analysis using 1  $\mu$ g/ml of the anti-heparan sulfate antibody (10E4). B – The cell lysates were treated with heparitinase I (Seikagaku Kogyo, Tokyo, Japan) for 1 h at 37°C. The digested lysate was electrophoresed on an 8.5% SDS-polyacrylamide gel and subjected to western blot analysis using 1  $\mu$ g/ml of the anti-neo-heparan sulfate antibody (3G10).

#### The increased expression of genes related to HS metabolism in the catalase transfectant clones

To investigate the relationship between the catalase and glycosyltransferases thought to affect HSPG synthesis, we studied the expression level of 24 selected genes related to HS metabolism in catalase transfectant clones by quantitative RT-PCR (Tab. 1). It was found that the HaCaT cells expressed 16 of the 24 genes selected. The level of HS2ST1 transcripts in the catalase transfectants was 2.7-fold higher than that in the control cells, and this was the most significant increase among all of the tested genes ( $P < 0.001$ ). The levels of HS6ST1 and GLCE transcripts were 4.5- and 1.8-fold higher than those in the



control cells ( $P < 0.01$ ). The levels of XT-II and EXTL2 transcripts were also significantly higher in the transfectants ( $P < 0.05$ ). HS3ST2 was the only enzyme with a significantly decreased level in the transfectants ( $P < 0.01$ ).

Tab. 1. The expression levels of the transcripts of various glycosyltransferases in the catalase transfectant clones.

	Catalase transfectant	Control	Relative to control
Catalase	7.2 ± 2.9**	2.1 ± 0.2	3.4
XT-I	0	0	
XT-II	0.16 ± 0.092*	0.049 ± 0.25	3.2
β4Gal-T7	0.44 ± 0.26	0.28 ± 0.33	1.6
β3Gal-T6	0.85 ± 0.5	0.61 ± 0.54	1.4
GlcAT-I	1.9 ± 0.83	1.0 ± 0.81	1.8
EXTL1	0	0	
EXTL2	0.60 ± 0.25*	0.32 ± 0.091	1.9
EXTL3	16 ± 9.1	9.8 ± 6.2	1.7
EXT1	2.4 ± 1.4	1.5 ± 1.1	1.6
EXT2	0.61 ± 0.36	0.51 ± 0.33	1.2
GLCE	5.8 ± 1.4**	3.3 ± 0.99	1.8
NDST1	4.0 ± 2.9	1.5 ± 0.23	2.6
NDST2	0.25 ± 0.26	0.12 ± 0.18	2.2
NDST3	0	0	
NDST4	0	0	
HS2ST1	0.69 ± 0.18***	0.26 ± 0.068	2.7
HS6ST1	2.7 ± 1.3**	0.59 ± 0.12	4.5
HS6ST2	0	0	
HS6ST3	0	0	
HS3ST1	0.99 ± 1.1	1.4 ± 1.3	0.7
HS3ST2	2.8 ± 1.6**	6.7 ± 1.6	0.4
HS3ST3	0.11 ± 0.1	0.11 ± 0.12	1.0
HS3ST4	0	0	
HS3ST5	0	0	

The amount of each glycosyltransferase transcript (copies/μl) was normalized to that of the β-actin transcripts ( $\times 10^3$  copies/μl). The values are the means ± SD. \* $P < 0.05$  compared to the control. \*\* $P < 0.01$  compared to the control. \*\*\* $P < 0.001$  compared to the control.

### The effect of hydrogen peroxide on the expression of genes related to HS metabolism

HaCaT cells were plated in a 6-well plate at a density of  $3 \times 10^5$  cells per well in 2 ml of growth medium, and cultured in complete culture medium with 20 or 100 μM hydrogen peroxide for 72 h. The levels of the HS2ST1 and HS6ST2

transcripts were significantly decreased by culturing with 20 or 100  $\mu\text{M}$  hydrogen peroxide ( $P < 0.01$ ; Fig. 4). The levels of XT-II, EXTL2 and GLCE were significantly decreased in the medium with 100  $\mu\text{M}$  hydrogen peroxide ( $P < 0.01$ ), and the level of GLCE transcripts was slightly down-regulated by 20  $\mu\text{M}$  hydrogen peroxide ( $P < 0.05$ ). By contrast, the level of HS3ST2 expression was significantly up-regulated in the medium with 100  $\mu\text{M}$  hydrogen peroxide ( $P < 0.01$ ).

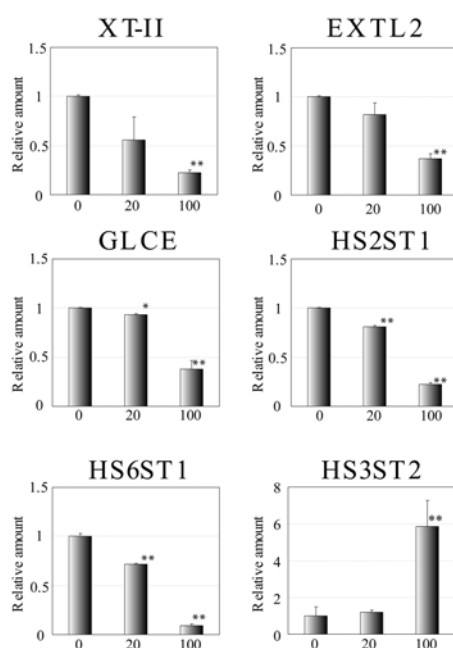


Fig. 4. The decreased expression of various genes related to HS metabolism in HaCaT cells due to culture with hydrogen peroxide. HaCaT cells were cultured in complete culture medium with 20 or 100  $\mu\text{M}$  hydrogen peroxide for 72 h. The level of each transcript was measured by quantitative RT-PCR. Each value is shown as a relative value compared to the normalized amount in the control. The values are the means  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ .

#### The decreased expression of the anti-heparan sulfate antibody epitope (10E4) in HaCaT cells due to the siRNA-mediated repression of catalase

HaCaT cells were transfected with Stealth RNAi targeted against the *catalase* gene (Fig. 5). The transfection efficiency ranged from 52 to 70%, and 24 h after transfection, the average efficiency was 61%. A competitive RT-PCR assay showed that introducing catalase-targeted Stealth RNAi into the HaCaT cells reduced the level of catalase mRNA by approximately 52% at 24 h and 27% at 48 h after transfection compared with the level in the control cells (Fig. 5B). The cells were subjected to two-color flow cytometric analysis to determine the expression levels of the anti-HS antibody (10E4) epitope 24 h after transfection.

The transfected cell population was gated by the higher intensity of orange fluorescent Alexa Fluor 546. The representative data from the FACS histograms showed that the rate of low HS staining was 20.2% in the cells transfected with Stealth RNAi (siRNA Catalase), but 15.1% in the control cells (siRNA Control; Fig. 5A). Thus, repressing catalase increased the population of cells with a low expression level of the 10E4 epitope. In addition, the level of HS2ST1 mRNA was reduced in the siRNA Catalase cells by approximately 33% compared with that in the control cells both 24 and 48 h after transfection (Fig. 5B). By contrast, the levels of other enzymes were not decreased by the transfection of this siRNA (data not shown); instead, the HS6ST1 level increased in the siRNA Catalase cells (Fig. 5B).

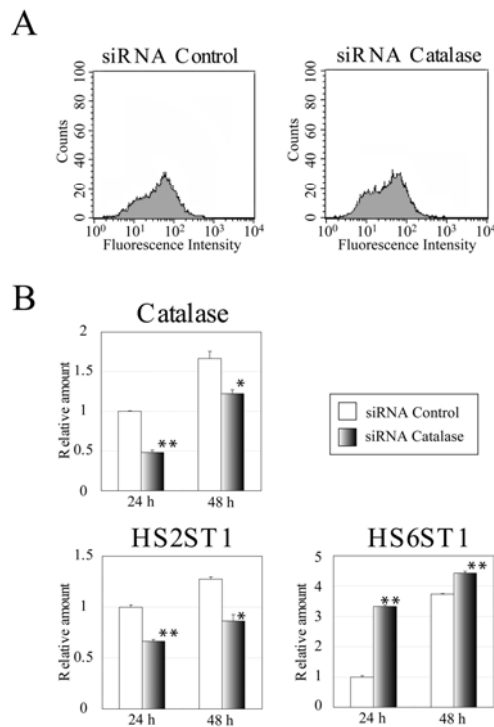


Fig. 5. The decreased expression of the anti-heparan sulfate antibody (10E4) epitope in HaCaT cells due to siRNA-mediated repression of catalase. HaCaT cells were transfected with Alexa Fluor 546 Stealth RNAi targeted against the catalase gene (siRNA Catalase) or the negative control Stealth RNAi (siRNA Control). A – The transfected cells were subjected to two-color FACS analysis 24 h after transfection. Representative FACS histograms using anti-HS antibody (10E4) are shown in orange fluorescent Alexa Fluor 546 positive cell populations. B – The levels of the catalase, XT-II, EXTL2, GLCE, HS2ST1, HS6ST1, and HS3ST2 transcripts were determined by quantitative RT-PCR 24 and 48 h after transfection and normalized to those of  $\beta$ -actin. Each value is shown as a relative value compared to the normalized amount of the control 24 h after transfection. The values are the means  $\pm$  SD. \* $P < 0.05$ ; \*\* $P < 0.01$ .

### The decrease in the cell growth rate in HaCaT cells due to the decrease in the catalase and HS2ST1 levels

To determine the role of the modification of HS chains in HaCaT cells, the cells were cultured with FGF7 after the repression of catalase (Fig. 6). Transfecting the HaCaT cells with Stealth RNAi targeted against the *catalase* gene (siRNA Catalase) reduced the cell proliferation rate. HaCaT cells expressed FGFR2-IIIb (data not shown), so FGF7 increased the rate of cell proliferation in a dose-dependent manner. However, the growth rate of the siRNA Catalase cells did not reach that of the control cells even after culture with 100 ng/ml of FGF7. Transfecting HaCaT cells with Stealth RNAi targeted against the *HS2ST1* gene (siRNA HS2ST1) also reduced the cell proliferation rate (Fig. 6B). FACS analysis showed that repressing HS2ST1 slightly increased the population of cells with a low expression level of the 10E4 epitope (data not shown), and the level of HS2ST1 mRNA was also reduced in siRNA HS2ST1 cells, by approximately 90% compared with that in the control cells both 24 and 48 h after transfection (data not shown).

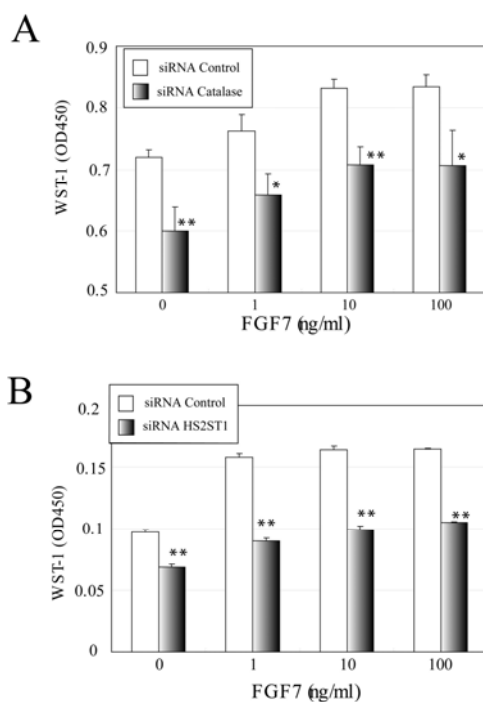


Fig. 6. The decrease in the cell growth rate in HaCaT cells due to repression of catalase. HaCaT cells were transfected with Stealth RNAi targeted against the *catalase* gene (siRNA Catalase; A), the *HS2ST1* gene (siRNA HS2ST1; B), or the negative control Stealth RNAi (siRNA Control). The cells were cultured in serum-free  $\alpha$ MEM with 1, 10 or 100 ng/ml FGF7 for 24 (A) or 48 h (B) after transfection. The WST-1 assay was performed after 24 h culture with FGF7. Values are the mean  $\pm$  SD. \* $P$  < 0.05; \*\* $P$  < 0.01.

## DISCUSSION

The biosynthesis of HSPG is a complex process involving the synthesis of core proteins and heparan sulfate chains. A heparitinase-digested lysate of HaCaT-Catalase cells contained almost the same level of the anti- $\Delta$ HS epitope of HSPG as a lysate of the control cells, implying that catalase does not affect the expression of the core proteins. However, western blotting detected the HS epitope as higher molecular weight molecules in the HaCaT-Catalase transfectants than in mock transfectants. The shift of smeared bands was compatible with the low mobility of highly glycosylated proteins in reducing SDS-PAGE gels.

Therefore, the increased expression of the anti-heparan sulfate antibody epitope (10E4) in the catalase transfectants appears to be due to increased modification of the HS chain rather than increased synthesis of the core proteins. Heparan sulfate chain synthesis is catalyzed by a number of enzymes, as described above. Each can catalyze a glycosyl transfer step and thereby supply the substrate of the next step, so that the reaction can proceed (Fig. 1). To identify important steps in this complex process, we examined the correlation between the expression of catalase and the respective enzymes in a number of clones of HaCaT-Catalase cells. Of these enzymes, the levels of XT-II, EXTL2, GLCE, HS2ST1 and HS6ST1 significantly increased in the catalase transfectants, whereas that of HS3ST2 decreased. XT-II was the only xylosyltransferase detected in the HaCaT cells by competitive RT-PCR (Tab. 1). The increase in the level of XT-II indicated that the activity of xylosyltransferase might have increased, and thus that the first glycosyl transfer step in the synthesis of HS might have been promoted in the catalase transfectants. The synthesis of HS is initiated by  $\alpha$ 1,4-*N*-acetylglucosaminyltransferase I (GlcNAcT-I), which can transfer GlcNAc to the common linkage, whereas the syntheses of chondroitin sulfate (CS) and dermatan sulfate (DS) are initiated by CSGalNAcT-1 and -2, which can transfer *N*-acetylgalactosamine (GalNAc) to the common tetrasaccharide through a  $\beta$ 1,4-linkage [27-29]. EXTL2 and EXTL3 have GlcNAcT-I activity, which initiates the synthesis of HS [30, 31].

HS chains can interact with FGFs possessing heparin-binding domains, and the FGF2-FGF receptor complex requires disaccharide units IdoA(2S)-GlcNS(6S) of HS chains to stabilize FGFR dimerization and activation [1]. GLCE, HS2ST2 and HS6ST1 can modify the backbone structure of HS, whereas XT-II and EXTL2 are involved in synthesizing the backbone. The modification of the HS backbone is a critical process in determining the function of HS chains. The three enzymes GLCE, HS2ST1 and HS6ST1 are essential for the formation of these disaccharide units. In particular, 2-*O*-sulfation has been shown to be an essential component of the high-affinity binding region within HS [32, 33]. Therefore, siRNA-mediated HS2ST1 repression decreased the cell growth rate in HaCaT cells. By contrast, HS3ST2 was inversely regulated compared to other enzymes like HS2ST1. The substrate specificity of HS3ST2 is similar to that of

HS6ST1 [34], so the distinct substrate preference might lead to competition between HS6ST1 and HS3ST2 if the availability of the acceptor substrates is limited. Up-regulation of HS2ST1 and HS6ST1 and down-regulation of HS3ST2 might co-operate to promote the synthesis of IdoA(2S)-GlcNS(6S).

The levels of five enzymes including HS2ST1 significantly increased in the catalase transfectants, whereas the level of HS3ST2 significantly decreased. By contrast, siRNA-mediated catalase repression decreased only the level of HS2ST1, but not that of other enzymes. The efficiency of catalase repression by siRNA was not high, although we made a lot of effort to find a good siRNA sequence. However, the HS2ST1 transcripts showed the most significant increase of all the tested genes in the case of catalase overexpression. Therefore, we supposed that HS2ST1 could most effectively respond to catalase-mediated hydrogen peroxide, whereas the low catalase repression may not have been effective enough to change the amount of the other enzymes. Adding enough hydrogen peroxide resulted in a change in the enzymes which were compatible with those of the catalase-overexpressing clones. Interestingly, the expression level of the anti-heparan sulfate antibody (10E4) epitope decreased even in siRNA-mediated catalase repression, as shown by the results of the FACS analysis. In addition, transient repression of catalase decreased the HaCaT cell proliferation, as did siRNA-mediated HS2ST1 repression. However, siRNA-mediated HS6ST1 repression did not decrease the HaCaT cell proliferation (data not shown). These findings revealed that HS2ST1 might be the essential gene in HaCaT cells to affect the affinity of receptors with FGFs by modifying HS chains in peroxide-mediated transcriptional regulation. On the other hand, it is known that both HS2ST1 and HS6ST1 have multiple substrate specificities of sulfotransferase in the synthesis of HS chains, but the relative activities of HS6ST1 for each substrate are not clear. Thus, if HS2ST1 is a bottleneck enzyme in the synthesis of HS chains, the level of HS2ST1 could be relatively important for the function of HS chains compared with the levels of other enzymes.

Judging by the effects of the addition of hydrogen peroxide, catalase might control the level of the gene expression related to HS metabolism through the regulation of hydrogen peroxide as a signaling molecule. There are several reports showing that hydrogen peroxide might suppress proteoglycan synthesis in the cartilage as a result of inflammation [35-37]. For example, the inhibition of catalase in chondrocytes was found to depress proteoglycan synthesis strongly, perhaps because the hydrogen peroxide level had increased inside the cells [35]. Hydrogen peroxide at 20 to 100  $\mu$ M could be cytotoxic to HaCaT cells; however, the viability of attached cells 72 h after the addition of hydrogen peroxide was estimated by staining with propidium iodine to be more than 95% (data not shown). In addition, the level of HS3ST2 expression was significantly up-regulated in the medium with 100  $\mu$ M hydrogen peroxide, in contrast to the other enzymes, which were down-regulated. These changes in the expression levels of enzymes were compatible with those in the catalase transfectant cells.

We showed here for the first time that hydrogen peroxide is able to modify HS chains through the transcriptional regulation of genes related to glycoconjugate metabolism in a keratinocyte cell line. We speculate that this mechanism is important in radiation-induced cutaneous fibrosis, the histological features of which induce the accumulation of extracellular components such as HSPG, because exposure to ionizing radiation results in the production of ROS in the lesion. Our findings revealed a novel association between ROS and GAGs. We demonstrated as a model that hydrogen peroxide played an important role as a regulator of the modification of HS chains in HaCaT cells through enzymes related to glycoconjugate metabolism. Accordingly, it was suggested that there might be some mechanisms of physiological regulation of HSPG by oxidative stress, and this theory might lead to further clarification of the mechanisms of the physiological and pathological events occurring in radiation injury. In addition, the availability of these enzymes might be useful for developing treatments for wounds and the protection of tissues against the radiation exposure that occurs in accidents or medical treatment by enhancing the effects of FGFs in the tissue.

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