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Expression of highly sulfated keratan sulfate synthesized in human glioblastoma cells

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Abstract

Keratan sulfate (KS) proteoglycan is expressed in the extracellular matrix or cell surface in numerous tissues, predominantly in those of the cornea, cartilage, and brain. However, its structure, function, and regulation remain poorly understood. Our investigation of KS expression in glioblastoma cell lines using Western-blot and flow cytometry with anti-KS antibody (5D4) revealed that LN229 glioblastoma cell highly expresses KS on a cell surface. Real-time PCR analysis showed that LN229 expresses a high level of keratan sulfate Gal-6-sulfotransferase. Results of this study also demonstrate that recombinant 5D4-reactive aggrecan is produced in LN229. Taken together, these results suggest that LN229 produces 5D4-reactive highly sulfated KS and is useful to investigate the KS structure and function in glioblastoma.

Keywords: Keratan sulfate; Proteoglycan; Glioblastoma; Glycogene

Keratan sulfate (KS) is a glycosaminoglycan that is formed through the elongation of *N*-glycans or *O*-glycans attached covalently to scaffold proteins [1]. So-called KS proteoglycans (KSPGs), which are KS-containing proteins, are found in the extracellular matrix or cell surface in numerous tissues, predominantly in those of the cornea, cartilage, and brain. In fact, KS expression responds to embryonic development, physiological variations, and wound healing [1–3].

Actually, KS consists of a linear polymer of *N*-acetyllactosamine, Galβ1-4GlcNAcβ1-3, which is sulfated at the C-6 positions of Galactose (Gal) and *N*-acetylglucosamine (GlcNAc) (Fig. 1A) [1]. Elongation of the carbohydrate backbone of KS chain is catalyzed by enzymes of two glycosyltransferase families: β1,3-*N*-acetylglucosaminyltransferase (β3GnT) and β1,4-galactosyltransferase (β4GalT). Sulfation of the chain is catalyzed by two carbohydrate sulfotransferases. Recent reports have described enzymes which are involved in KS synthesis [4–10]. Both β3GnT7 and β4GalT4 are responsible for KS chain elongation. Keratan sulfate Gal-6-sulfotransferase (KSGal6ST) is for sulfation of Gal. In addition, *N*-acetylglucosamine-6-*O*-sulfotransferase (GlcNAc6ST)-1 and GlcNAc6ST-5 (also known as CGn6ST) are, respectively, responsible for sulfation of GlcNAc in the brain and cornea. Results of *in vitro* studies indicate that KS synthesis occurs in two stages: the production of GlcNAc-sulfated poly-*N*-acetyllactosamine chain by β3GnT7, β4GalT4, and GlcNAc6ST-5; and the production of highly sulfated KS through sulfation of Gal by KSG6ST [4,5,10].

Abbreviations: KS, keratan sulfate; KSPGs, KS proteoglycans; KSGal6ST, keratan sulfate Gal-6-sulfotransferase; GlcNAc6ST, *N*-acetylglucosamine-6-*O*-sulfotransferase; β3GnT, β1,3-*N*-acetylglucosaminyltransferase; β4GalT, β1,4-galactosyltransferase; Gal, Galactose; GlcNAc, *N*-acetylglucosamine.

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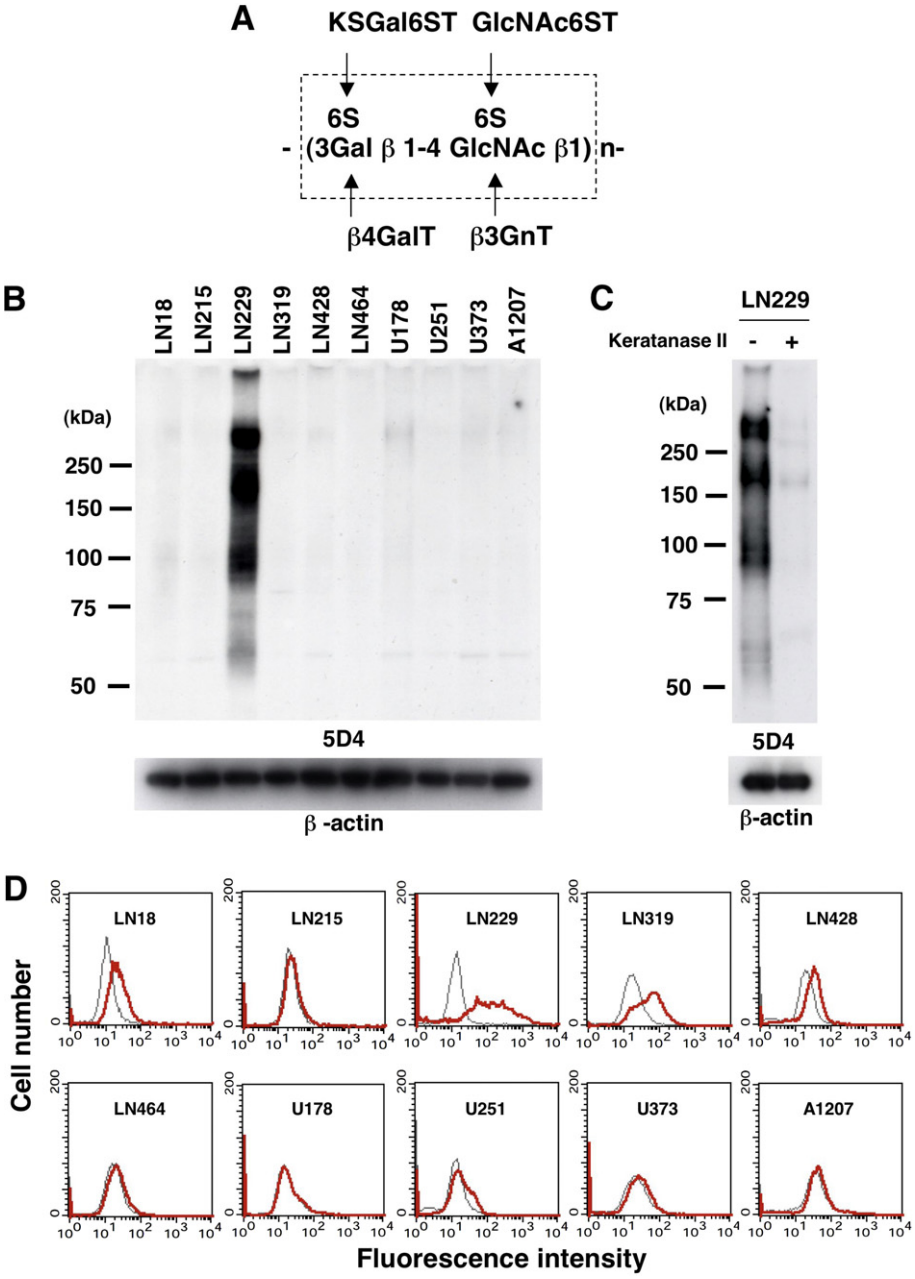

Fig. 1. Analysis of KS expression in 10 glioblastoma cell lines. (A) Structure and synthesis of KS. (B) Cell lysates of 10 glioblastoma cells were electrophoresed and immunoblotted using anti-KS (5D4) or anti-β-actin. (C) The LN229 lysates treated with keratanase II were analyzed using Western-blot with 5D4 or anti-β-actin. (D) Flow cytometric analyses of glioblastoma cell lines were performed using 5D4.

Most cells do not produce KS in culture because KS-specific sulfotransferases are down-regulated in culture conditions [10,11]. Primary bovine keratocytes cultured in serum-free media continue KS synthesis *in vitro* at reduced rates and with less sulfation: production of recombinant KSPGs to analyze biological function of KS is not efficient [12–14]. Furthermore, immortal cells have not been reported to produce high levels of KS.

Results of this study show that one glioblastoma cell, LN229, highly expresses KS using a monoclonal anti-KS antibody (5D4). Furthermore, we investigated the relationship between expression of KS and glycogenes that are involved in KS synthesis using quantitative real-time PCR in 10 glioblastoma cell lines. Results also show that the LN229 cells produce KS-containing proteoglycans carrying both *N*-linked and *O*-linked KS both endogenously and exogenously.

Materials and methods

Cell lines and reagents. The glioblastoma cell lines [15] were donated by Dr. Webster K. Cavenee (Ludwig Institute for Cancer Research, San Diego, CA), and cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in Dulbecco's modified Eagle's medium (DMEM; Invitrogen

Corp., Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO) and 1% of penicillin–streptomycin solution (Invitrogen Corp., Carlsbad, CA).

Western-blot analysis. The cell lines were lysed with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA) including protease inhibitor solution (Sigma). Samples of the supernatant fraction were collected after centrifuging at 15,000*g* for 30 min. Two micrograms of the proteins was electrophoresed under reducing conditions on 10% polyacrylamide gel (Atto Bioscience, Tokyo, Japan). The separated proteins were transferred to a PVDF membrane. After blocking with 3% skim milk in PBS, the membrane was incubated with 5D4 (1/5000 dilution; Seikagaku Corp., Tokyo, Japan) or anti-β-actin antibody (1/5000 dilution; Sigma), and subsequently with peroxidase-conjugated anti-mouse antibodies (1/5000 dilution; Bio-Rad Laboratories Inc., Hercules, CA). It was then developed for 1 min with ECL reagents (Amersham Pharmacia Biotech Inc.) using Amersham Hyperfilm ECL (Amersham Pharmacia Biotech Inc.).

Flow cytometry. The cell lines that were collected using EDTA treatment (10 mM EDTA in PBS) were incubated with 5D4 (1/1000 dilution) for 1 h at 4 °C. Then the cells were incubated with Oregon green-conjugated antibody (Invitrogen Corp.) for 30 min. Flow cytometry was performed using FACS Calibur (BD Biosciences, Barintree, MA).

Deglycosylation experiments. The LN229 cell lysate was treated with keratanase II (0.25 mU/μg of protein, 10 mM sodium citrate, pH 6.2; Seikagaku Corp.) for 12 h at 37 °C or PNGase F (0.1 mU/μg of protein; Takara Bio Inc., Shiga, Japan) for 24 h at 37 °C according to the manufacturer's instructions. Alkaline digestion of *O*-glycans on the Western-blot was performed as described [16]. Briefly, transferred membrane of LN229 cell lysate was incubated with 0.05 N NaOH for 16 h at 40 °C, prior to probing with antibody.

Quantitative real-time PCR analysis. Total RNAs were prepared from glioblastoma cell lines using an RNeasy mini prep kit (Qiagen Inc., Hilden, Germany). The initial cDNA strand was synthesized using SuperScript III transcriptase (Invitrogen Corp.) by priming nine random oligomers and an oligo-dT primer according to the manufacturer's instructions. Real-time PCR was carried out on a LightCycler 480 Instrument with 96-well unit (Roche, Mannheim, Germany), using the LightCycler 480 SYBR Green I Master (Roche). Sets of primers were designed online with Primer3 (http://frodo.wi.mit.edu). The PCR conditions were 95 °C for 5 min (1 cycle), followed by 45 cycles of 95 °C for 10 s, 65 °C for 20 s, 72 °C for 20 s. Subsequently, a melting curve program was applied with continuous fluorescence measurement. Standard curves for each glycogene and β-actin template were generated by serial dilution of the PCR products (1×10^8 to 1×10^2 copies/μl). The expression levels of glycogenes were normalized by the copy number of β-actin.

RNAi experiments. Small interfering stealth RNAs (siRNAs) for KSGal6ST were purchased from Invitrogen Corp. The target sense sequences of KSGal6ST siRNA #1, #2, and #3 were 5′-AGGGCAA GUACAUGUUGGUGCGCUA-3′, 5′-ACCUGGCUCGGAACCCUA UGAAGAA-3′, and 5′-AGCACAAAUACGGCACCGUGCGAAA-3′, respectively. As control siRNA, stealth RNAi negative control duplex (Invitrogen Corp.) was used. The LN229 cells were transfected with 25 nM siRNA using LipofectamineRNAiMAX (Invitrogen Corp.) in accordance with the manufacturer's protocol. After 3 days, total RNAs were prepared; after 4 days, cell lysates of transfected cells were prepared and analyzed as described above.

Expression of KSGal6ST. To construct an expression vector for KSGal6ST, we amplified cDNA encoding the KSGal6ST ORF by PCR using BC022567 (Open Biosystems, Huntsville, AL) as the template, along with the following primers: 5′ primer, 5′-CACCATGCAATGTT CCTGGAAG-3′ and 3′ primer, 5′-TCACGAGAAGGGGCGGAAGT-3′. The PCR product was phosphorylated with T4 polynucleotide kinase (New England Biolabs Inc., Ipswich, MA) and cloned into the EcoRV site of pcDNA3.1 (Invitrogen Corp.). The generated plasmid was designated as pcDNA3.1/KSGal6ST. The LN464 cells were transfected with pcDNA3.1/KSGal6ST or empty vector using FuGENE6 (Roche) in accordance with the manufacturer's protocol. After 3 days, the cell lysates of transfected cells were prepared and analyzed.

Expression of FLAG-tagged human aggrecan G1–G2. To construct an expression vector for FLAG-tagged human aggrecan G1–G2 (rAggrecanG1–G2) [12,17], we amplified cDNA encoding the G1–G2 domain using PCR with BC036445 (Open Biosystems) as the template and the following primers: 5′ primer containing EcoRI site 5′-gcgaattcatcgATGACCA CTTTACTCTGGGT-3′ and M13 reverse primer 5′-caggaaacagctatgac-3′. The PCR product was digested with EcoRI and BamHI, and cloned into the EcoRI and BglII sites of pFLAG-CMV14 (Sigma). The generated plasmid was designated as pFLAG-CMV14/rhG1–G2. The LN229 cells were transfected with 15 μg of pFLAG-CMV14/rhG1–G2 using FuGENE6 (Roche). After three days, the conditioned medium was immunoprecipitated using 20 μl of anti-FLAG (M2) agarose (Sigma), and the rAggrecanG1–G2 proteins were eluted with 100 μl of 0.2 mg/ml 3xFLAG peptide (Sigma). Subsequently, 5 μl of the eluted protein was undigested or digested with keratanase II (0.1 mU/5 μl of the eluted protein) for 12 h at 37 °C or PNGase F (0.25 mU/5 μl of the eluted protein) for 24 h at 37 °C, followed by Western-blot analysis using 5D4 or peroxidase-conjugated anti-FLAG antibody (M2: 1/4000 dilution; Sigma).

Results and discussion

KS expression in glioblastoma cell lines

The brain is reported to be one of tissues, which highly expresses KS [1,3]. To investigate the expression of KS in glioblastoma, we performed Western-blot analysis against 10 glioblastoma cell lines using monoclonal anti-KS antibody, 5D4, which recognize highly sulfated KS [18]. As shown in Fig. 1B, KS was highly expressed in one glioblastoma cell line, LN229. Keratanase II treatment of LN229 cell lysate markedly decreased the 5D4-reactivity observed using Western-blot analysis (Fig. 1C). Next, the KS expression on cell surface was investigated using flow cytometry with 5D4. Results showed that LN229 cells expressed KS on a cell surface at a higher level than did other glioblastoma cell lines (Fig. 1D). Although KS expression in other human tumor cell lines was also examined, a high level of KS expression was not observed (data not shown).

Comparison of expression patterns of five genes involved in KS synthesis in glioblastoma cell lines

We next performed quantitative real-time PCR analysis to compare the respective expression levels of five genes involved in KS synthesis in 10 glioblastoma cell lines: KSGal6ST, GlcNAc6ST-1/-5, β3GnT7, and β4GalT4. Fig. 2 shows that the clearest difference between LN229 and the others was that of KSGal6ST expression: LN229 cells expressed KSGal6ST at higher levels compared to other cell lines (Fig. 2A). Furthermore, LN229 cells expressed GlcNAc6ST-1/-5 at higher levels (Fig. 2B and C); β3GnT7 is expressed in LN229 at the highest level of all cell lines (Fig. 2D).

Effect of expression of KSGal6ST on expression of 5D4-reactive KS in LN229 and LN464 cells

We performed knockdown of KSGal6ST in LN229 using small interference RNAs (siRNAs) for KSGal6ST

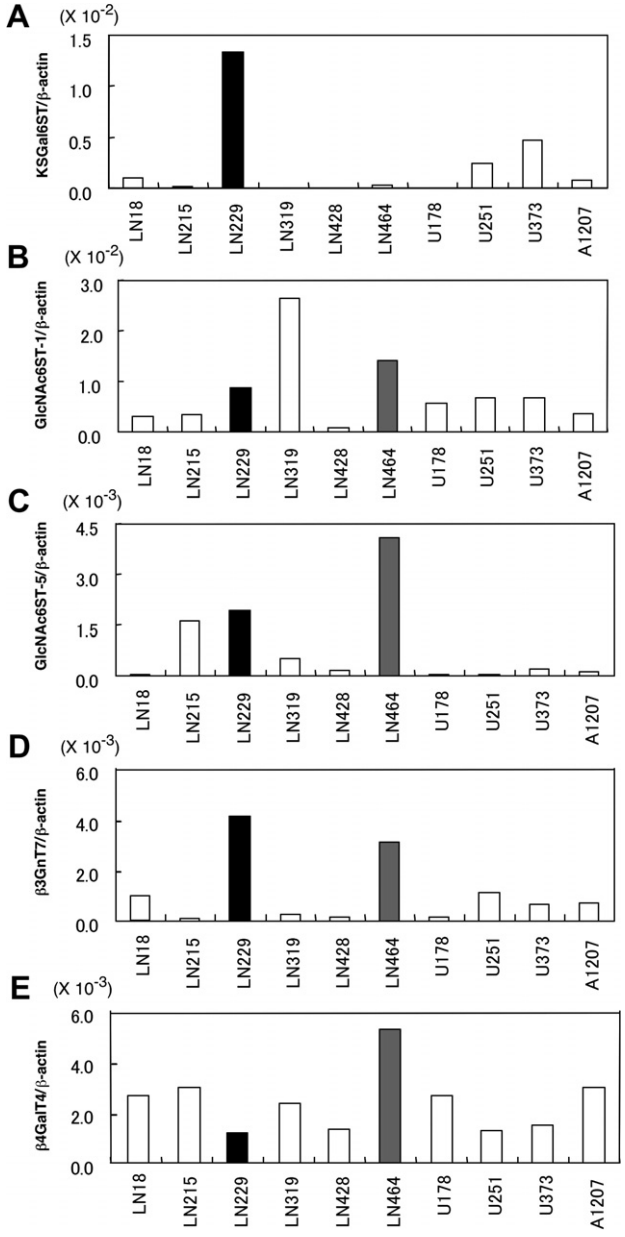

Fig. 2. Quantitative real-time PCR analysis of glycogenes for KS synthesis. The transcript levels for (A) KSGal6ST, (B) GlcNAc6ST-1, (C) GlcNAc6ST-5, (D) β3GnT7, and (E) β4GalT4 genes in each cell line were measured using real-time PCR. Values normalized to the level of β-actin transcripts are presented.

to investigate the importance of KSGal6ST in expression of highly sulfated KS. The siRNAs for KSGal6ST decreased the expression level of KSGal6ST transcripts (data not shown). Furthermore, siRNAs for KSGal6ST considerably reduced the 5D4-reactivity shown by Western-blot analysis compared to negative control siRNA (Fig. 3A). As presented in Fig. 2, another glioblastoma cell, LN464, expresses higher level of GlcNAc6ST-1/-5 and β4GalT4 and an equal level of β3GnT7 in comparison to LN229. However, LN464 expresses a much lower level of KSGal6ST. We transfected KSGal6ST into LN464 cells to clarify the importance of KSGal6ST for expression of

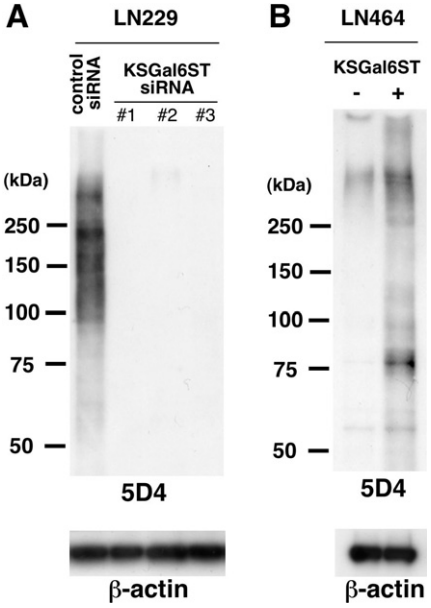

Fig. 3. Effect of KSGal6ST on KS synthesis in LN229 and LN464 cells. (A) siRNAs for KSGal6ST (#1, #2, and #3) were transfected into LN229 cells. Cell lysates of LN229 cells were electrophoresed and immunoblotted using anti-KS (5D4) or anti-β-actin. (B) KSGal6ST gene was transfected into LN464 cells. Cell lysates of LN464 cells were electrophoresed and immunoblotted using anti-KS (5D4) or anti-β-actin.

highly sulfated KS. Results show that, KSGal6ST-transfected LN464 expresses a higher level of highly sulfated KS than control LN464 (Fig. 3B). These results indicate that KSGal6ST is necessary for 5D4-reactive, highly sulfated KS in glioblastoma cells.

Production of N-linked and O-linked KS-containing proteoglycans by LN229 cells

To determine whether LN229 cells express *N*-linked or *O*-linked KS, the LN229 cell lysate was digested with PNGase F, then analyzed by Western-blot using 5D4. As depicted in Fig. 4A, the PNGase F treatment reduced most 5D4-positive signals. However, several signals were still observed. The PNGase F-undigested signals disappeared almost completely by sequential alkaline hydrolysis treatment to remove *O*-glycans (Fig. 4A), indicating that LN229 cells express both *N*-linked and *O*-linked KS. Next, we examined whether *N*-linked and *O*-linked KS are attached to recombinant proteins, such as a G1–G2 fragment of aggrecan in LN229 cells. As presented in Fig. 4B, immunoprecipitated rAggrecanG1–G2 was detected with 5D4. The 5D4 signals were decreased by keratanase II treatment, although rAggrecanG1–G2 was detected using anti-FLAG antibody. Subsequent PNGase F treatment partially decreased the molecular size of rAggrecanG1–G2. However, most 5D4-reactivity was not affected. Furthermore, PNGase F-undigested signals disappeared completely by alkaline hydrolysis (Fig. 4C). These

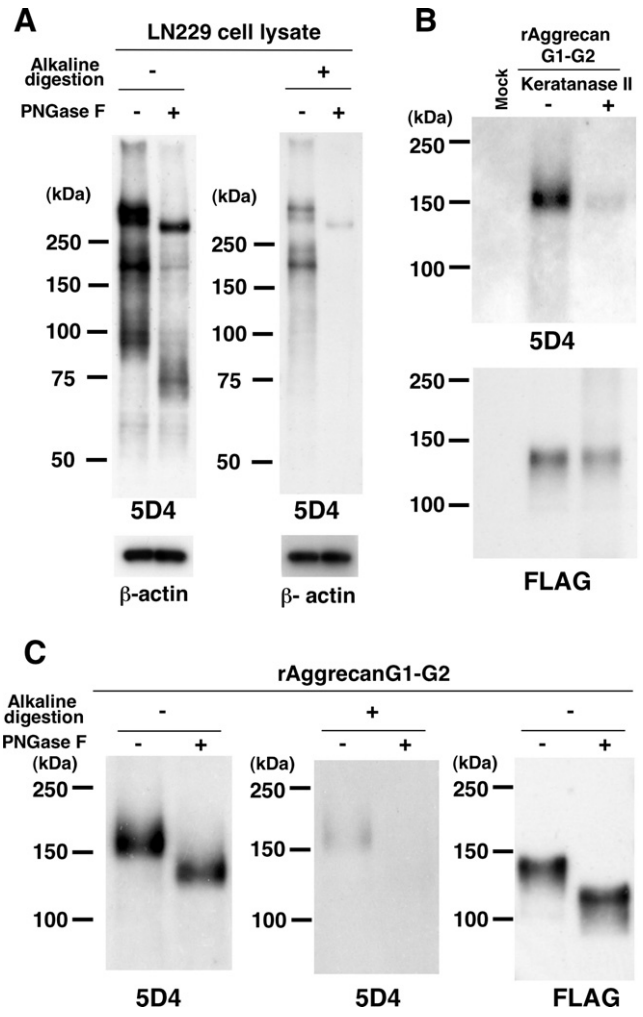

Fig. 4. Analysis of KS-linkages of KSPGs produced by LN229 cells. (A) LN229 cell lysates after treatment with PNGase F were electrophoresed and transferred onto PVDF membranes. The membranes were treated with PBS (left panel) or 0.05 M NaOH (right panel), and immunoblotted with 5D4 or anti-β-actin. (B) LN229 cells were transfected with rAggrecan G1-G2 or mock; these conditioned media were immunoprecipitated using anti-FLAG. Immunoprecipitates were treated using keratanase II, and were immunoblotted using 5D4 (upper panel) or anti-FLAG (lower panel). (C) Immunoprecipitates using anti-FLAG were treated with PNGase F, electrophoresed and transferred onto PVDF membranes. The membranes were treated with PBS (left panel and right panel) or 0.05 M NaOH (middle panel), and immunoblotted with 5D4 (left panel and middle panel) or anti-FLAG (right panel).

results suggest that LN229 produce rAggrecanG1–G2 with both *N*-linked and *O*-linked KS.

In summary, we showed that a glioblastoma cell line, LN229, strongly expresses highly sulfated KS. Experiments of knockdown and over-expression of KSGal6ST showed that KSGal6ST is essential for 5D4-reactive, highly sulfated KS synthesis. Deglycosylation treatments showed that LN229 express both *N*-linked and *O*-linked KS-containing proteoglycans endogenously and exogenously. These results suggest that LN229 might be useful to investigate the biological activities and the structure of KS *in vivo*. Now, we are investigating the expression of KS in clinical glioblastoma tissues, and obtained the preliminary results indicating that KS expression is associated with that malignancy. Further study is necessary to investigate whether KS is involved in the invasion of glioblastoma or its malignant progression.

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