MATRIX PATHOBIOLOGY

Microglial Keratan Sulfate Epitope Elicits in Central Nervous Tissues of Transgenic Model Mice and Patients with Amyotrophic Lateral Sclerosis

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The functional role of 5D4 antibody-reactive keratan sulfate (KS) in the pathogenesis of neurodegenerative diseases is unknown. We therefore studied the expression of 5D4-reactive KS in amyotrophic lateral sclerosis (ALS), a motor neuron-degenerative disease, with the use of SOD1^{G93A} ALS model mice and patients with ALS. Histochemical and immunoelectron microscopic characterizations showed that the 5D4-reactive KS is expressed in Mac2/galectin-3–positive activated or proliferating microglia of SOD1^{G93A} ALS model mice at disease end stage and that the KS is an *O*-linked glycan modified with sialic acid and fucose, which was thus far shown to exist in cartilage. Intriguingly, microglial KS was detected in the spinal cord and brainstem but not in the cerebral cortex of SOD1^{G93A} mice. We found that KSGal6ST, a galactose-6-sulfotransferase, is required for biosynthesis of the microglial 5D4-reactive KS by generating SOD1^{G93A}/KSGal6ST^{−/−} mice. The requirement of GlcNAc6ST1 for this synthesis was corroborated by analyzing SOD1^{G93A}/GlcNAc6ST1^{−/−} mice. These results indicate that both galactose-6– and *N* acetylglucosamine-6–sulfated KS elicited in the spinal cord and brainstem are associated with the degeneration of spinal and bulbar lower motor neurons in ALS pathology and may play a role in disease progression via microglial activation and proliferation. (*Am J Pathol* 2015, 185: 3053–3065; <http://dx.doi.org/10.1016/j.ajpath.2015.07.016>)

Glycan structures often show characteristic differences between a normal state and disease. Knowledge of disease-specific glycan structures can help to understand the mechanisms underlying disease progression and to develop new diagnostic and therapeutic approaches. Keratan sulfate (KS) is an extracellular polysaccharide classified as a member of the glycosaminoglycans. It was originally isolated from the cornea.¹ KS is also found in skeletal and nervous tissues.^{2,3} In terms of the composition of KS proteoglycans, one or more KS chains are covalently attached to a core protein through the elongation of *N*-linked, *O*-*N*-acetylgalactosamine (GalNAc)-linked or *O*-mannose–linked oligosaccharides.^{4,5} KS is composed of repeating disaccharides of galactose (Gal) and *N*-acetylglucosamine (GlcNAc), with sulfate groups at their C-6 positions.^{2,6} The capping structures at their nonreducing termini vary and include modifications with α 3- or α 6-linked sialic acid, α 3-linked Gal, or β 3-linked sulfated GalNAc.^{7,8} Golgi-resident enzymes catalyze sulfation modifications and

elongation of the KS disaccharides, producing diversity in the chains' structure. The C-6 sulfation modifications in KS synthesis are dictated by the Gal/GlcNAc/GalNAc sulfotransferases.⁹ Among those, GlcNAc6ST1, GlcNAc6ST3, GlcNAc6ST5, and KS galactose-6-sulfotransferase (KSGal6ST) (encoded by the *Chst2*, *Chst5*, *CHST6*, and *Chst1* genes, respectively) were reported to mediate KS sulfation *in vivo*.^{10–12}

Several monoclonal antibodies against KS were used to evaluate KS expression.^{13–16} 5D4 recognizes KS oligosaccharide structures with absolute dependence on both Gal- and GlcNAc-6-sulfation modifications.^{11,12,17,18}

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However, additional glycosylation modifications of the 5D4-reactive KS glycan and the precise structure of the 5D4 epitope still remain to be determined. 5D4 was raised initially against human articular cartilage proteoglycans.¹³ In central nervous system tissues, the expression of the 5D4-reactive KS is induced in rodent models after injury.^{18,19} We have shown that treatment with keratanase II, a KS-degrading bacterial enzyme, or deficiency in the *Chst2* gene increased axonal sprouting and regeneration in injured nervous tissues.^{20,21} Expression of the 5D4 epitope was also found physiologically in the developing brain of rat and mouse.^{12,22,23} Expression of the 5D4 epitope in microglia is extensively induced with neurodegeneration in mouse models of cerebral amyloid angiopathy,^{24,25} familial Danish dementia,²⁶ and light-induced retinal degeneration.²⁷ However, the functional role of 5D4-reactive KS in the pathogenesis of neurodegenerative disease is unknown.

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease that attacks spinal and bulbar motor neurons (lower motor neurons) in the spinal cord and brainstem and corticospinal and corticobulbar motor neurons (upper motor neurons) in the cerebral cortex, leading to progressive muscle weakness and complete paralysis within 1 to 5 years after disease onset.²⁸ The mutation of expanded GGGGCC hexanucleotide repeat in the *C9ORF72* gene is the most common cause of ALS. The repeat expansions are related to up to 40% of familial ALS and 10% of sporadic ALS in the United States and Europe.²⁹ Mutations in the gene encoding copper–zinc superoxide dismutase (*SOD1*) are found in 10% to 20% of familial ALS and up to 5% of sporadic ALS globally.^{30,31} The *SOD1*^{G93A} transgenic mouse is an ALS model mouse.³² This mouse develops an ALS-like phenotype that includes loss of motor neurons in the spinal cord by the disease end stage.³² It was reported that the lower and upper motor neurons degenerate independently of each other and that these degenerative processes are associated with intense inflammatory reactions in the spinal cord and brain.³³ New methods to monitor the progression of degenerative changes in the lower and upper motor neurons will help our understanding of causative mechanisms underlying ALS and aid to develop therapeutic strategies. Recently, we have shown that the 5D4 epitope is induced in microglia in the spinal cord of *SOD1*^{G93A} mice with disease progression and that GlcNAc6ST1 is involved in 5D4-reactive KS synthesis.³⁴ It was unclear whether the microglial 5D4-KS glycan is elicited in the brainstem and cerebral cortex of *SOD1*^{G93A} mice. The sulfotransferase responsible for Gal-6-sulfation within the 5D4-KS in microglia of *SOD1*^{G93A} mouse has remained unknown. Here, we report that the microglial 5D4-KS consists of sialyl-, fucosyl-, O-linked glycans, and that the 5D4-KS is expressed in the brainstem but not in the cerebral cortex of *SOD1*^{G93A} mice. We have also found that KSGal6ST is an enzyme required for Gal-6-sulfation within the microglial 5D4-reactive KS in the spinal cord and brainstem of *SOD1*^{G93A} mice, with a

corroborating correlation between the expression level of GlcNAc6ST1 and the 5D4 epitope.

Materials and Methods

Mice

C57BL/6J mice were purchased from SLC Inc. (Hama-matsu, Japan). ALS model B6.Cg-Tg(SOD1*G93A)1Gur/J (*SOD1*^{G93A}) mice³² obtained from The Jackson Laboratory (Bar Harbor, ME) were provided by Dr. Akio Suzumura, Nagoya University, Japan. The generation of GlcNAc6ST1-deficient (*GlcNAc6ST1*^{−/−}) or KSGal6ST-deficient (*KSGal6ST*^{−/−}) mice was as previously described.^{35,36} Males of *SOD1*^{G93A} hemizygotes were bred with *GlcNAc6ST1*^{−/−} or *KSGal6ST*^{−/−} females. Generated *SOD1*^{G93A}/*GlcNAc6ST1*^{+/-} or *SOD1*^{G93A}/*KSGal6ST*^{+/-} males were then bred with *GlcNAc6ST1*^{+/-} or *KSGal6ST*^{+/-} females. The mice were kept under controlled environmental conditions and were provided with standard nourishment and water. All experimental procedures were approved by the Animal Research Committee of Nagoya University and conducted in accordance with the guidelines of Nagoya University.

Colony Maintenance of the *SOD1*^{G93A} Transgenic Mice

Colony maintenance and experimental tests for the *SOD1*^{G93A} transgenic mice and the transgenic progeny were performed in accordance with guidelines provided by The Jackson Laboratory. The *SOD1*^{G93A} transgene was genotyped with the genomic DNA isolated from a 0.5-cm section of the tail. Isolation of tail DNA was performed by a sodium hydroxide extraction method. The tail section cut into small pieces was mixed with 180 µL of 100 mmol/L NaOH. The sample was incubated at 95°C for 10 minutes, cooled to room temperature, and then neutralized with 20 µL of 1 mol/L Tris-HCl (pH 8.0). The sample was centrifuged at 15,000 × *g* for 10 minutes at 4°C. The supernatant fluid was subjected to PCR reactions. A total of 25 µL of the PCR mixture contained 0.25 µL of the tail DNA, 12.5 µL of 2× PCR buffer for KOD FX, 0.4 mmol/L of each dNTP, 5 µmol/L of each primer, 2.5% (v/v) dimethyl sulfoxide, and 0.5 units of KOD FX DNA polymerase (Toyobo, Osaka, Japan). The thermal cycling was set as follows: 94°C for 1.5 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and then 4°C until the samples were subjected to 1.5% agarose gel electrophoresis. The primers for the transgene (mutated *SOD1*) were 5'-CATCAGCCCTAATCCATCTGA-3' (forward) and 5'-CGCGACTAACAATCAAAGTGA-3' (reverse).³⁷ Copy numbers of the transgene were determined with the use of real-time quantitative PCR (qPCR) in accordance with The Jackson Laboratory protocol run on Light Cycler 480 (Roche Diagnostics, Mannheim, Germany). The transgene copy number was determined by comparing the ΔCt value of each sample against a standard high-copy control and a low-copy control, with the use of appropriate endogenous references. All primers

described in The Jackson Laboratory protocol were obtained from MBL (Nagoya, Japan). High-copy number transgenic mice were subjected to experimental analyses. Low-copy number transgenic mice were euthanized.

Antibodies and Enzymes

The following materials were obtained from the commercial sources indicated. 5D4 anti-KS monoclonal antibody was from Seikagaku (Tokyo, Japan); rabbit anti-GlcNAc6ST-1/CHST2 antibody was from Sigma-Aldrich (St. Louis, MO); mouse anti-KSGal6ST/CHST1 antibody (clone A-2) was from Santa Cruz Biotechnologies (Dallas, TX); biotinylated rat anti-mouse Mac-2 was from Cedarlane (Burlington, NC); rabbit anti-Iba1 antibody was from Wako (Osaka, Japan); horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 was from Caltag (Burlingame, CA); HRP-conjugated goat anti-mouse IgG2b, cyanine 2 (Cy2)-conjugated streptavidin, Cy3-conjugated goat anti-mouse IgG1, and Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) were from Jackson ImmunoResearch (West Grove, PA); and HRP-conjugated goat anti-rabbit IgG (H+L) was from Cell Signaling Technology (Danvers, MA). Enzymes used for digestion were α 2-3,6,8 neuraminidase (*Arthrobacter ureafaciens*) from Nacalai Tesque (Kyoto, Japan); α 1-3,4-fucosidase (*Streptomyces* sp.) from Takara Bio Inc. (Shiga, Japan); keratanase II (*Bacillus* sp.) from Seikagaku; peptide-N-glycosidase F (PNGase F; *Flavobacterium meningosepticum*) from Roche Diagnostics (Indianapolis, Indiana); and α 1-2 fucosidase (*Xanthomonas manihotis*), recombinant α -N-acetylgalactosaminidase, and α 1-3,6 galactosidase (*Xanthomonas manihotis*) from New England Biolabs (Ipswich, MA).

Human Postmortem Spinal Cord Tissues

Patients with sporadic ALS received a pathologic diagnosis according to the El Escorial Diagnostic World Federation of Neurology criteria (Table 1). The diagnosis of ALS was histopathologically confirmed by the presence of intraneuronal Bunina bodies. Specimens of cervical (C3 to C6 segments) and lumbar spinal cords (L4 to L5 segments) from ALS cases and disease control cases were obtained. These specimens were frozen and then stored at -80°C . The collection of tissues, their use in this study, and the consent procedure were approved by the Ethics Committee of Nagoya University Graduate School of Medicine. The written informed consent was obtained from the patients' next-of-kin. Among the specimens examined, those with preserved expression of β -actin were subjected to evaluation of KS expression. Frozen spinal cords were homogenized with a Dounce tissue grinder in Tris-buffered saline (TBS; 20 mmol/L Tris and 137 mmol/L NaCl, pH 7.6) containing 1% Triton X-100 and protease inhibitors (complete protease inhibitor cocktail; Roche Diagnostics) and then used for biochemical analyses.

Table 1 Clinical Information of ALS Disease and Non-ALS Control Donor Patients Used in the Western Blot Analysis of the 5D4 Keratan Sulfate Epitope

Patient number	Age, year	Sex	Disease	ALS disease duration, year
ALS disease patients				
ALS-230	61	F	ALS	5
ALS-231	66	M	ALS	2.7
ALS-233	53	F	ALS	2.7
ALS-260	66	M	ALS	3
Non-ALS controls				
Ctrl-138	74	F	MSA-P	
Ctrl-177	77	M	PSP	
Ctrl-216	84	M	MG	

ALS, amyotrophic lateral sclerosis; F, female; M, male; MG, myasthenia gravis; MSA-P, multiple system atrophy with predominant parkinsonism; PSP, progressive supranuclear palsy.

Mouse Tissues

Mice were anesthetized and transcardially perfused with phosphate-buffered saline (PBS). Spinal cords and brains were dissected out and divided into cross-sectional segments and sagittal parts, respectively. Hemi-brains and segments of cervical, thoracic, and lumbar spinal cords were embedded in a compound for frozen sectioning. Thoracic and lumbar parts of spinal cords and regional parts of hemi-brains, namely, brainstem, thalamus, hippocampus, cerebellum, olfactory bulb, and the frontal motor region of cerebral cortex, were separated on ice, snap-frozen, and then stored at -80°C for biochemical analysis.

Isolation of Primary Microglia from Adult Mice

Microglia were isolated from whole spinal cords of adult SOD1^{G93A} and wild-type mice as previously described³⁸ with minor modifications. SOD1^{G93A} littermate mice with end-stage disease were deeply anesthetized and perfused transcardially with ice-cold PBS. Spinal cords were dissected out, cut into fragments with a scalpel, and then incubated with an enzyme solution that contained 5 mg/mL collagenase and 10 $\mu\text{g}/\mu\text{L}$ DNase I (Roche Diagnostics) for 45 minutes at 37°C . The tissue was gently disrupted by pipetting several times with a 1-mL pipette, filtered through a 70- μm cell strainer, and then centrifuged at $190 \times g$ for 10 minutes. The cell pellet was suspended with myelin magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) to remove myelin debris. The cells were then pelleted and re-suspended with PBS supplemented with 0.5% bovine serum albumin (BSA), 2 mmol/L EDTA, and CD11b magnetic beads (Miltenyi Biotec) for 15 minutes at 4°C . The suspension was subjected to magnetic-activated cell sorting selection columns according to the manufacturer's instructions. CD11b⁺ cells were collected and suspended in Dulbecco's modified Eagle's/F12 medium supplemented with 10% heat-inactivated fetal

bovine serum. The cell suspension was seeded on a 6-well plate coated with poly-L-lysine (Sigma-Aldrich) and maintained in culture at 37°C. The medium was replaced every 2 days. Collected CD11b⁺ cells were characterized by qPCR, immunocytochemistry, and Western blot analysis. CD11b⁺ cells in flow-through fractions on the mass spectrometry column were also collected and analyzed.

Fractionation of Spinal Cord and Brain Samples

Snap-frozen spinal cords and various regions of brains (approximately 20 mg) were taken in a 1.5-mL tube and supplemented with 600 μ L (30 volumes of the tissue weight) of ice-cold TBS with protease inhibitors as previously described.³⁹ The tube was then placed in the water bath of a Bioruptor sonicator (Cosmo Bio, Tokyo, Japan). Tissues were fragmented for 15 seconds with the maximum ultrasonic wave output four to five times until solid materials in the tube became invisible. The materials were then ultra-centrifuged at 100,000 $\times g$ for 30 minutes at 4°C. The supernatant fluid was collected and stored frozen as the TBS-soluble fraction. The resulting pellet was suspended in 600 μ L (the same volume) of TBS that contained 1% SDS, and the pellet was dissociated and centrifuged at 15,000 $\times g$ for 20 minutes at room temperature. The resulting supernatant fluid was collected and stored frozen as the TBS-insoluble/1% SDS-soluble fraction. The protein concentrations of both fractions were measured by the Bradford method.

Enzymatic Treatment and Solvolysis

Snap-frozen spinal cords were homogenized in TBS that contained 1% Triton X-100 and protease inhibitors. Supernatant fluids were collected after centrifugation at 10,000 $\times g$ for 30 minutes at 4°C. The protein concentration was measured by the Bradford method. For pretreatment, the samples were digested with 0.05 U/mL neuraminidase and/or 1 U/mL α -1,3/4-L-fucosidase at 37°C for 2 hours. Then, the enzymatic reaction was stopped by heating the samples at 95°C for 5 minutes. The samples were then treated with 0.05 U/mL keratanase II at 37°C overnight. Digestion of the samples with 1 U/mL PNGase F or β -elimination (GlycoProfile β -elimination kit; Sigma-Aldrich), α 1-2 fucosidase, recombinant α -N-acetylgalactosaminidase, or α 1-3,6 galactosidase was performed according to the manufacturer's instructions. Solvolysis was performed as described previously.⁴⁰ Fourteen microliters (10 μ g of protein) of each TBS-insoluble/1% SDS-soluble fraction of SOD1^{G93A} spinal cord was dried *in vacuo*. A 14- μ L solution of 0.05 N methanolic HCl was prepared with the Methanolic HCl kit (Supelco Analytical, Bellefonte, PA) and then added to the dried materials. The materials were incubated at 4°C to avoid influences of sialic acids within KS or at room temperature for 4 hours and then mixed with 1.4 μ L of 0.1 N NaOH. The materials were dried *in vacuo* and then subjected to immunoblot analyses.

Immunoblots

The proteins (20 μ g) were separated with 7.5% polyacrylamide gel electrophoresis (SuperSep; Wako) and blotted onto a polyvinylidene difluoride membrane (GE Healthcare, Little Chalfont, United Kingdom). The membrane was blocked with 5% skim milk in PBS that contained 0.1% Tween-20 for 1 hour at room temperature and then incubated at 4°C overnight with primary antibody diluted in 5% skim milk in that contained 0.1% Tween-20. The next day, the membrane was washed several times with that contained 0.1% Tween-20 and incubated with HRP-conjugated secondary antibody at room temperature for 1 hour. Bound antibodies were visualized with chemiluminescent reagent Pierce Plus (Thermo Scientific) and autoradiography. Immunoblotting was performed with the following antibody concentrations: anti-KS 5D4 (dilution 1:1000), anti-GlcNAc6ST-1/CHST2 (dilution 1:1000), and anti-KSGal6ST/CHST1 (dilution 1:1000). Secondary antibodies were HRP-conjugated goat anti-mouse IgG1 (dilution 1:3000), goat anti-rabbit IgG (H+L) (dilution 1:1000), and goat anti-mouse IgG2b (dilution 1:5000), respectively. An anti- β -actin antibody (dilution 1:10,000) was used as a loading control. The relative intensity of immunoreactive bands was quantified by calculating pixel densities of scanned images of the X-ray film. Densitometry analysis was performed in a blinded manner (T.F.) on a Macintosh computer with the use of the ImageJ version 1.44o (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). Levels of 5D4-KS, GlcNAc6ST1, and KSGal6ST were normalized to the levels of β -actin to control for protein loading.

Immunohistochemistry

Tissues were embedded in the Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA). Frozen tissues were cut into 10- μ m sections on a cryostat and collected on MAS-coated glass slides (SF17293; Matsunami, Osaka, Japan), fixed in ice-cold acetone for 15 minutes and then allowed to dry for 30 minutes. Sections were incubated with blocking solution (3% BSA in PBS) for 15 minutes at room temperature, washed twice with PBS, and then incubated with a mixture of 5D4 (dilution 1:80) and biotinylated rat anti-mouse Mac-2 (dilution 1:200) or a mixture of 5D4 (dilution 1:80) and rabbit anti-Iba I antibody (dilution 1:200) at 4°C overnight. The next day, after washing with PBS, the sections were incubated with a mixture of Cy3-conjugated goat anti-mouse IgG1 (dilution 1:250) and Cy2-conjugated streptavidin (dilution 1:250) or a mixture of Cy3-conjugated goat anti-mouse IgG1 and Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (dilution 1:250) for 60 minutes at room temperature, rinsed, and counterstained with Hoechst. Then, the sections were mounted with FluorSave (Merck, Darmstadt, Germany). Digital images were captured by fluorescence microscopy (model BX41 microscope; Olympus, Tokyo, Japan). Digital images at randomly selected five fields within the

ventral horn area of three spinal cord sections (three mice for each genotype) and within the brainstem of three brain sections (three mice for each genotype) were captured in a blinded manner (K.U., T.F.). When a Mac2⁺ or an Iba1⁺ cell showed the 5D4-KS staining signals that surrounded the staining signal of Mac2 or Iba1, we categorized the cell as a 5D4⁺/Mac2⁺ or 5D4⁺/Iba1⁺ cell. Hoechst staining confirmed the cell body. The number of these stained cells was counted and quantitated. Immunostaining with an isotype-matched control (mouse IgG1, biotinylated rat IgG2a, or rabbit IgG) for each primary antibody gave no specific signals in the SOD1^{G93A} spinal cord or brainstem (data not shown).

Immunocytochemistry

BV-2 and N9 microglia cells were provided by Dr. Tony Wyss-Coray, Stanford University, Stanford, CA. The cells were transfected with pCMV6 Entry Myc/DDK-mChst1 and/or pCMV6-mChst2 as described previously.¹² Forty-eight hours later, the cells were washed with PBS and then fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. Sections were incubated with 3% BSA in PBS for 30 minutes at room temperature for blocking and then incubated with 5D4 anti-KS antibody (dilution 1:500) overnight at 4°C. Cells were washed three times with PBS, and bound antibody was detected with Cy3-anti-mouse IgG1 antibody (dilution 1:250). Images were captured with a fluorescence microscope.

Immunoelectron Microscopy

Mice were anesthetized with 50 mg pentobarbital/kg (Dainippon Sumitomo Pharma, Osaka, Japan) and then transcardially perfused with PBS and 4% paraformaldehyde/0.05% glutaraldehyde. Brains were dissected out, fixed overnight in 4% paraformaldehyde/0.05% glutaraldehyde, and then sank sequentially in 10%, 15%, 20%, and 30% sucrose in PBS. Thirty-micron coronal brain sections were cut with a freezing microtome and then stored in a cryoprotective medium. Sections were blocked with PBS that contained 3% BSA for 1 hour at room temperature. Sections were incubated with 5D4 (dilution 1:50) in PBS that contained 0.3% BSA overnight at 4°C. Sections were then incubated with 1.4 nm Nanogold-conjugated goat anti-mouse IgG (H+L) (Nanoprobes, Yaphank, NY) overnight at 4°C. Sections were fixed again with 2% glutaraldehyde in 0.1 mol/L phosphate buffer, followed by enlargement of the gold particles with the Gold Enhance EM Formulation (Nanoprobes). Sections were then postfixed with 2% osmium tetroxide in 0.1 mol/L cacodylate buffer overnight at 4°C. The specimens were examined in a JEM-1400 Plus transmission electron microscope (JEOL Ltd., Tokyo, Japan).

qPCR

Total RNA was extracted from frozen mouse tissues and primary microglia with the use of TRIzol Reagent (Invitrogen,

Carlsbad, CA) according to the manufacturer's instructions. Total RNA (5 to 7 µg) was subjected to reverse transcription in 100 µL of buffer with random primers, using Superscript II Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed on Mx3000P (Agilent Technologies, Santa Clara, CA) with the use of SYBR qPCR mix (Toyobo Co. Ltd., Osaka, Japan). PCR was performed with the following variables: 95°C for 10 seconds, 1 cycle and then 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. The relative expression levels of each mRNA were calculated with the $\Delta\Delta C_t$ method, normalizing to the level of *Gapdh*. Conclusions were drawn from triplicate PCR reactions. The primer sequences used in this study are newly designed and were confirmed by using samples of mouse peripheral lymph nodes.⁴¹ The primers are as follows: for GlcNAc6ST1, 5'-GT-ATGGCCAAGACGCTGCAA-3' (forward) and 5'-AGATC-CTCGTACCTCACCACCAAG-3' (reverse); for GlcNAc6ST2, 5'-TCAAGTTCAAGGCGTGTCTGGTA-3' (forward) and 5'-CCGTTCCACAGGTCAGCATC-3' (reverse); for GlcNAc6ST3, 5'-TGGTGCCTACGAGGATCTG-3' (forward) and 5'-TGTGGATCCAAGTCTGGAGCTG-3' (reverse); for GlcNAc6ST4, 5'-AGTATTGCAAGTTCACGCTGCTC-3' (forward) and 5'-CTCGCTGTGGCTGTCCAGTA-3' (reverse); for C6ST1, 5'-TGTTCTCGCTGCGATTTGTGGTCATA-3' (forward) and 5'-CCAACCTCGCTCAGGGACAAGA-3' (reverse); for KSGal6ST, 5'-CTGACAGATACTGGCTTG-GCAGA-3' (forward) and 5'-GACATCCATGTGCTGGTT-GAAGA-3' (reverse); for β 1,4-galactosyltransferase 4, 5'-ACCTGCTGGAACACCTGCATC-3' (forward) and 5'-GTTTCAGGAGCTTGGCTCGGTTA-3' (reverse); for β 1,3-N-acetylglucosaminyltransferase 7 (β 3Gnt7), 5'-TCCC-AGCCGTCATGTATGGTAA-3' (forward) and 5'-ATGGTG-GAGTTGCCGAGCTAA-3' (reverse); for β -1,3-N-acetylglucosaminyltransferase 2 (β 3Gnt2), 5'-TCTGGTCTCAG-TTGCAAAGTCCTAA-3' (forward) and 5'-GGCTACCTG-CTCATGAAGGCTAA-3' (reverse); for Axl, 5'-GGAGGA-GCCTGAGGACAAAGC-3' (forward) and 5'-TACAGC-ATCTTGAAGCCAGAGTAGG-3' (reverse); for insulin-like growth factor 1, 5'-CCTCTGGGATACGGCACTTA-3' (forward) and 5'-GAAGGTCTTGGTGGCATGTT-3' (reverse); for IL-1 β , 5'-CCTGCAGCTGGAGAGTGTGGAT-3' (forward) and 5'-TGTGCTCTGCTTGTGAGGTGCT-3' (reverse); for tumor necrosis factor- α , 5'-AGCCCACGTCGTAGCAAAC-CAC-3' (forward) and 5'-AGGTACAACCCATCGGCTG-GCA-3' (reverse); and for transforming growth factor- β , 5'-TACGGCAGTGGCTGAACCAA-3' (forward) and 5'-CGGTTTCATGTCATGGATGGTG-3' (reverse).

Statistical Analysis

All data are presented as means $\pm$ SD unless otherwise noted. The values were analyzed by unpaired Student's *t*-test or one-way analysis of variance with Dunnett's test with Prism software version 6 (GraphPad Software, La Jolla, CA). *P* < 0.05 was considered statistically significant. Differences in survival times were analyzed with Kaplan-Meier survival statistics

Figure 1 5D4-reactive keratan sulfate expressed in spinal cords of SOD1^{G93A} mice consists of sialylated, fucosylated *O*-glycans. **A:** TBS-insoluble/1% SDS-soluble fractions were prepared from spinal cords of 24-week-old (disease end-stage) SOD1^{G93A} mice. Western blot analysis with 5D4 and quantitative analysis measuring the relative intensities of bands indicated by an open arrowhead were performed. **B:** Immunoreactivity of 5D4 on SOD1^{G93A} spinal cord homogenates after solvolytic desulfation reaction. **C:** One percent Triton X-100-soluble fractions of SOD1^{G93A} spinal cords were pretreated with α 2-3,6,8 neuraminidase (sialidase), α 1-3,4 fucosidase, or both at 37°C for 2 hours. Subsequently, the reaction mix was treated with keratanase-II at 37°C overnight. One percent Triton X-100-soluble fractions were pretreated with endo- β -galactosidase at 37°C for 2 hours (**D**) or with α 1-2 fucosidase, GalNAcase, or α 1-3,6 galactosidase (**E**). The treated samples were subjected to Western blot analysis with 5D4. **F:** One percent Triton X-100-soluble fractions of SOD1^{G93A} spinal cords were incubated with PNGase F. TBS-insoluble/1% SDS-soluble fractions were subjected to β -elimination. The immunoreactivity of the 5D4 was tested. $n = 3$ (non-Tg and SOD1^{G93A} mice). **** $P < 0.0001$. GalNAcase, α -N-acetylgalactosaminidase; PNGase F, peptide-N-glycosidase F; TBS, Tris-buffered saline; Tg, transgenic.

(log-rank test; SPSS Software version 23, IBM Japan, Tokyo, Japan).

Results

Characterization of 5D4-Reactive KS

In SOD1^{G93A} mice, significant death of motor neurons in the spinal cord occurs at the onset of clinical disease (at 12 weeks old). The mice show up to 50% loss of cervical and lumbar motor neurons by disease end stage (20 to 24 weeks old).³² To investigate the expression of the 5D4 KS epitope and sulfotransferase in the SOD1^{G93A} ALS model mice in the end-stage and in their age-matched nontransgenic littermates, we performed Western blot analysis for spinal cord samples, which were fractionated as TBS-insoluble/1% SDS-soluble. One band of high intensity corresponding to a size of 150 kDa and at least two bands of lower intensity with sizes of 200 kDa and 250 kDa were immunoreactive for 5D4 (Figure 1A). Quantitative analysis by measuring the intensities of these bands by densitometry revealed that there was a 22-fold increase in the intensity of overall 5D4 recognition determinants in SOD1^{G93A} spinal cord extracts compared with that in the nontransgenic controls (Figure 1A). Desulfation of the SOD1^{G93A} lysate samples by solvolysis at room temperature and at 4°C led to a substantial reduction in the 5D4 immunoreactivity (Figure 1B). We have reported that the

treatment of keratanase II, an endo-type KS-degrading enzyme, diminished the 5D4 immunoreactivity in injured spinal cords.²⁰ To determine whether the 5D4 epitope in the spinal cord of SOD1^{G93A} mice is susceptible to keratanase II, spinal cord lysates (1% Triton X 100-soluble fractions) of 24-week-old SOD1^{G93A} mice were treated with the enzyme. Unexpectedly, we observed that the 5D4 immunoreactivity remained after keratanase II treatment (Figure 1C). Certain classes of KS are modified with sialic acid and fucose.^{42,43} We determined whether there are glycosylation modifications that could interfere with the keratanase II activity on the 5D4-reactive KS chains. We pretreated lysate samples of SOD1^{G93A} spinal cords with α 2-3,6,8 neuraminidase (sialidase), α 1-3,4 fucosidase, or both, followed by keratanase II digestion. We found a mobility shift of the major 150 kDa band in the case of the neuraminidase treatments. The major band was shifted to 130 kDa (Figure 1C). Susceptibility of the 150-kDa major band to keratanase II was not altered by α 2-3,6,8 neuraminidase single pretreatment, as judged by almost the same level of 5D4 immunoreactivity remaining. α 1-3,4 Fucosidase treatment showed mobility of the major band comparable with that on no treatment. Interestingly, the band became smeared (Figure 1C). When the lysate was pretreated with a mixture of the neuraminidase and the fucosidase, and subsequently treated with keratanase II, the 5D4 immunoreactivity was reduced to an undetectable level (Figure 1C). A comparable level of 5D4 immunoreactivity was

Figure 2 5D4-reactive keratan sulfate is expressed in Mac⁺ microglia. **A:** Cryostat-cut spinal cord sections of 24-week-old (disease end stage) SOD1^{G93A} mice were co-stained with 5D4 (red) and anti-Mac2 (green) or anti-Iba1 (green) antibody. Representative images of 5D4⁺Mac2⁺ (arrowheads), 5D4⁻Mac2⁺ (arrow), and 5D4⁺Iba1⁺ (open arrowheads) cells are shown. Graphs are of semiquantitative analysis of immunohistochemical images with Hoechst nuclear staining. Fractions of 5D4⁺ and 5D4⁻ cells among Mac2- or Iba1-expressing cells were calculated as described in [Materials and Methods](#). **B:** CD11b⁺ primary microglia isolated from SOD1^{G93A} spinal cords at the disease end stage were cultured for 4 days *in vitro* and stained with 5D4 (red) or Iba1 (green) antibody. Immunocytochemistry confirmed these isolated primary microglia to be Iba1⁺ (98%) or 5D4⁺ (90%). Representative images of fluorescently stained cells are shown. One percent Triton X-100-soluble fractions were prepared and immunoblotted with 5D4. *****P* < 0.0001. Scale bar = 25 μm.

retained, but the major band slightly shifted to 140 kDa on treatment with endo-β-galactosidase, another KS-degrading enzyme (Figure 1D). Treatment of the lysate with α1-2 fucosidase, α-N-acetylgalactosaminidase, or α1-3,6 galactosidase did not alter the 5D4 immunoreactivity (Figure 1E). Next, to determine whether the 5D4-reactive KS is linked through N-glycan or not, a 1% Triton X-100-soluble lysate of SOD1^{G93A} spinal cords was incubated with PNGase F and then subjected to Western blot analysis. The 5D4 immunoreactivity to the treated sample was comparable with that of the control sample (Figure 1F). β-Elimination reaction abolished the 5D4 immunoreactivity in the sample (Figure 1F).

5D4-Reactive KS Is Expressed in Mac2/Galectin3-Positive Microglia

We previously showed that the 5D4 epitope is elicited in both CD86⁺ and CD86⁻ microglia in SOD1^{G93A} spinal

cords at the disease end stage.³⁴ To determine whether the 5D4 epitope is induced in activated and proliferating microglia, we stained SOD1^{G93A} spinal cord sections with an antibody against Mac2, a marker for activated and proliferating myeloid cells, including microglia.⁴⁴ We found that the Mac2 signals (90%) and the signals of Iba1 (96%), a microglia marker, were mostly colocalized with 5D4 signals (Figure 2A). Microglial expression of the 5D4-reactive KS was substantiated by signals seen in immunocytochemical staining and Western blot analysis in 4-day *in vitro*-cultured CD11b⁺ primary microglia isolated from spinal cords of SOD1^{G93A} end-stage mice (Figure 2B). CD11b magnetic bead-selected microglia from spinal cords were subjected to quantitative PCR analyses for genes of proinflammatory cytokines and neuroprotective factors for motor neurons. The level of mRNA expression of IL-1β significantly increased (11-fold) in SOD1^{G93A}/GlcNAc6ST1^{-/-} primary microglia but not in SOD1^{G93A}/KSGal6ST^{-/-} microglia

Figure 3 Sulfotransferases required for 5D4-reactive KS expressed in the spinal cord of SOD1^{G93A} mice. **A:** Total RNA from the spinal cords of 24-week-old non-Tg and SOD1^{G93A} end-stage mice were prepared and tested. mRNA levels of Gal/GlcNAc/GalNAc sulfotransferases and KS glycosyltransferases were determined by quantitative real-time PCR. IGF1 and Axl were analyzed as described previously.³⁷ **B:** TBS-insoluble/1% SDS-soluble fractions were prepared from tissue homogenates of 24-week-old non-Tg and SOD1^{G93A} end-stage mice. Western blot analysis was performed with anti-GlcNAc6ST1 and anti-KSGal6ST antibodies. Relative intensities of bands indicated by open arrowheads were measured by densitometry as described in *Materials and Methods*. **C:** The spinal cord fractions were prepared from 24-week-old SOD1^{G93A}, SOD1^{G93A}/GlcNAc6ST1^{-/-}, and SOD1^{G93A}/KSGal6ST^{-/-} mice. Western blot analysis was performed. β-Actin was used as an internal loading control. **D:** Kaplan-Meier curve analysis shows a shortened median survival time of SOD1^{G93A}/GlcNAc6ST1^{-/-} mice (158 ± 1.3 days) compared with SOD1^{G93A} mice (169 ± 1.7 days) ($P = 0.006$, log-rank test). Comparable median survival times of SOD1^{G93A} mice (168 ± 5.1 days) and SOD1^{G93A}/KSGal6ST^{-/-} mice (172 ± 1.9 days) are shown. All mice are on a C57BL/6J genetic background. **E:** Mouse microglia cells, BV-2 and N9, were transfected with cDNA of an empty vector (Mock), mouse GlcNAc6ST1, KSGal6ST, or both (GlcNAc6ST1/KSGal6ST). Cells were stained with 5D4 (red) and Hoechst (blue), and then visualized with a fluorescence microscope. Representative images of 5D4-expressing cells (arrowheads) are shown. Data are expressed as medians ± SE. $n = 3$ (**A** and **B**, non-Tg mice and SOD1^{G93A} end-stage mice); $n = 7$ (**D**, SOD1^{G93A}/GlcNAc6ST1^{-/-} mice); $n = 12$ (**D**, SOD1^{G93A} mice); $n = 15$ (**D**, SOD1^{G93A} and SOD1^{G93A}/KSGal6ST^{-/-} mice). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bar = 25 μm. Gal, galactose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; IGF, insulin-like growth factor; KS, keratan sulfate; TBS, Tris-buffered saline; Tg, transgenic; KSGal6ST, keratan sulfate galactose-6-sulfotransferase.

compared with those from SOD1^{G93A} mice (Supplemental Figure S1). The level of mRNA expression of tumor necrosis factor-α significantly increased (1.5- to 2.0-fold) in both SOD1^{G93A}/GlcNAc6ST1^{-/-} and SOD1^{G93A}/KSGal6ST^{-/-} primary microglia. Interestingly, mRNA expression levels of transforming growth factor-β (5.7-fold) and insulin-like growth factor 1 (1.9-fold) significantly increased in SOD1^{G93A}/KSGal6ST^{-/-} primary microglia (Supplemental Figure S1). Such up-regulation was not observed in SOD1^{G93A}/GlcNAc6ST1^{-/-} microglia.

Sulfotransferases Required for 5D4-Reactive KS in SOD1^{G93A} Mice

To understand possible mechanisms of up-regulation of the 5D4 epitope in the spinal cord of the ALS model mouse, we measured the mRNA expression levels of six Gal/GlcNAc/GalNAc sulfotransferases and three glycosyltransferases by qPCR. These are regarded as key enzymes in KS synthesis. The levels of mRNA expression of GlcNAc6ST1 and β3GnT7 significantly increased (3.5- to 4-fold) in

Figure 4 5D4-reactive KS is induced in the brainstem but not in the cerebral cortex of SOD1^{G93A} mice. **A:** Brain specimens of SOD1^{G93A}, SOD1^{G93A}/GlcNAc6ST1^{-/-}, and SOD1^{G93A}/KSGal6ST^{-/-} mice at the disease end stage were stained with 5D4 antibody (red). Nuclear staining was performed with Hoechst (blue). **B:** Cryostat-cut brainstem sections of 24-week-old (disease end stage) SOD1^{G93A} mice were co-stained with 5D4 (red) and anti-Mac2 (green) or anti-Iba1 (green) antibody. Graphs are of semi-quantitative analysis of immunohistochemical images. Fractions of 5D4⁺ and 5D4⁻ cells among Mac2- or Iba1-expressing cells in the brainstem were calculated as described in *Materials and Methods*. **C:** Pre-embedding immunoelectron microscopy for the 5D4 epitope in the brainstem of the SOD1^{G93A} mouse at disease end stage. The nuclear membrane lined with dense heterochromatin is a distinguishing feature of microglia. 5D4 signals along with cell surface membrane structures of microglia and microglial processes are shown (**open arrow-heads**). 5D4 signals found in membrane structures in microglial contacts with a dendrite are indicated (**arrows**). **D:** TBS-insoluble/1% SDS-soluble fractions were prepared from brainstem, thalamus, cerebellum, cerebral cortex, hippocampus, and olfactory bulb of SOD1^{G93A}, SOD1^{G93A}/GlcNAc6ST1^{-/-}, and SOD1^{G93A}/KSGal6ST^{-/-} mice. Western blot analysis was performed with 5D4 and antibodies against GlcNAc6ST1, KSGal6ST, and β -actin. **** $P < 0.0001$. Scale bars: 45 μ m (**A**), 25 μ m (**B**), and 1 μ m (**C**). d, dendrite; KS, keratan sulfate; n, nucleus; p, microglial perikaryon; KSGal6ST, keratan sulfate galactose-6-sulfotransferase; GlcNAc, *N*-acetylglucosamine; TBS, Tris-buffered saline.

SOD1^{G93A} spinal cords, whereas those of other sulfo-transferases and glycosyltransferases were comparable with those of the nontransgenic controls (**Figure 3A**). Consistent with a report by Chiu et al⁴⁵ that mRNA expression levels of insulin-like growth factor 1 and Axl receptor tyrosine kinase were up-regulated in SOD1^{G93A} spinal cords at the disease end stage, the levels of expression of these genes were increased 4.5-fold (**Figure 3A**). Immunoblotting was performed for TBS-insoluble/1% SDS-soluble fractions of spinal cord samples with anti-KSGal6ST and anti-GlcNAc6ST1 antibodies under nonreducing conditions. We found that the expression level of the GlcNAc6ST1 protein was increased 20-fold in SOD1^{G93A} mice, whereas that of KSGal6ST was comparable with the level of the nontransgenic mice (**Figure 3B**).

We then investigated whether the induced expression of 5D4-reactive KS could account for the expression of GlcNAc6ST1 and KSGal6ST in the SOD1^{G93A} spinal cord. We generated SOD1^{G93A}/GlcNAc6ST1^{-/-} and SOD1^{G93A}/KSGal6ST^{-/-} mice. We corroborated that the disruption of GlcNAc6ST1 abolished the 5D4 epitope in the spinal cord (**Figure 3C**). Clearly, SOD1^{G93A}/KSGal6ST^{-/-} spinal cords did not show expression of the 5D4 epitope at the end stage (**Figure 3C**).

As we observed in the previous report,³⁴ a life span of SOD1^{G93A}/GlcNAc6ST1^{-/-} mice was significantly shortened compared with SOD1^{G93A} mice (**Figure 3D**). SOD1^{G93A}/KSGal6ST^{-/-} mice showed a life span similar to that of SOD1^{G93A} mice (**Figure 3D**). We investigated whether GlcNAc6ST1 and KSGal6ST are sufficient to elaborate the

5D4 epitope in mouse microglial cells. BV-2 and N9 cells were transfected with pcDNA3.1, pCMV6-Entry Myc/DDK-mChst1, pCMV6-mChst2, or both pCMV6-Entry Myc/DDK-mChst1 and pCMV6-mChst2.¹² The transfection efficiency was measured by counting the percentage of green-fluorescent cells in parallel experiments with pcDNA3-EGFP expression plasmid. The efficiency was determined to be 10% in BV-2 and N9 cells. Immunostaining by 5D4 showed that approximately 3% of BV-2 and N9 cells transfected with cDNAs for both GlcNAc6ST1 and KSGal6ST were positive for the stained signals (Figure 3E). Cells transfected with either cDNA alone did not show expression of the 5D4 epitope (Figure 3E).

5D4-Reactive KS Is Induced in the Brainstem but Not in the Cerebral Cortex of SOD1^{G93A} Mice

Next, we investigated whether the 5D4-reactive KS might also be expressed in the brain of SOD1^{G93A} mice. Immunohistochemical analysis and Western blot analysis revealed that 5D4-reactive KS is abundantly expressed in the brainstem but not in the frontal motor cortex of SOD1^{G93A} mice at disease end stage (Figure 4, A, B, and D). As seen in the spinal cord, 5D4 signals were mostly co-stained with the Mac2 signals (95%) and the Iba1 signals (99%) (Figure 4B). A moderate level of the 5D4 signal was seen in the white matter of deep cerebellum nucleus (Figure 4, A and D). These prominent signals were not observed in either SOD1^{G93A}/GlcNAc6ST1^{-/-} or SOD1^{G93A}/KSGal6ST^{-/-} mice (Figure 4, A and D). We performed immunoelectron microscopy with 5D4. 5D4⁺ signals were seen in cell surface membrane structures of microglia and their processes (Figure 4C).

Elicited 5D4-Reactive KS in Postmortem Spinal Cords of ALS Patients

To test if the elicited expression of the 5D4 KS epitope in SOD1^{G93A} ALS model mice was also observed in ALS patients, we performed Western blot analysis for postmortem cervical and lumbar spinal cords of disease-control and sporadic ALS patients (Table 1), which were fractionated as 1% Triton X-100 soluble. All four ALS cervical spinal cords showed 5D4-immunoreactive smear bands with molecular weights of >400 kDa and >250 to 150 kDa. These bands were not detectable in all three disease control specimens (Figure 5). Two of the four ALS lumbar spinal cords showed 5D4-immunoreactive smear bands similar to those found in the cervical (Figure 5).

Discussion

In the present study, the structure of the 5D4-reactive KS was characterized. We found that the KS glycans expressed in the spinal cord of SOD1^{G93A} mice at the end stage are

Figure 5 Expression of 5D4-reactive keratan sulfate up-regulated in spinal cords of ALS patients. One percent Triton X-100-soluble fractions were prepared from postmortem cervical and lumbar spinal cords of disease control and sporadic ALS patients. Proteins (20 μ g/lane) were loaded in each lane (Table 1). Western blot analysis with 5D4 and an anti- β -actin antibody was performed as described in *Materials and Methods*. $n = 3$ (disease control); $n = 4$ (ALS). ALS, amyotrophic lateral sclerosis.

produced as molecules of multiple sizes and that the 5D4 antibody does indeed recognize the sulfated moiety, as indicated by an apparent reduction in the 5D4 immunoreactivity after solvolysis. The 5D4-reactive KS expressed in the spinal cord of SOD1^{G93A} mice is resistant to keratanase II but becomes susceptible to it on pretreatment of the lysate sample with a neuraminidase and a fucosidase. It can be deduced that the presence of sialic acid and fucose residues within the 5D4-reactive KS glycans protects the KS against keratanase II by interfering with the endo-type cleavage. The poor susceptibility to endo- β -galactosidase well supports this prediction, as previously reported.^{46,47} These results indicate that 5D4-reactive KS is capped with sialic acids at its nonreducing terminus and composed of C6-sulfated GlcNAc residues that are modified with α 3-linked fucoses and that the 5D4-reactive KS is rather short, speculated to be composed of Sia α 2-3Gal(6S) β 1-4GlcNAc(6S) β 1-3Gal β 1-4(α 1-3Fuc)GlcNAc(6S) β 1-6R or -2R (the R denotes GalNAc or Man).

The absence of a clear change in the mobility and immunoreactivity of the 5D4-reactive bands on pretreatment with α 1-2 fucosidase, α -N-acetylgalactosaminidase, or α 1-3,6 galactosidase suggested that the 5D4-reactive KS glycans are not modified with α 2-linked fucose, nonreducing α -GalNAc, or α 3-linked Gal. The susceptibility to β -elimination but not PNGase F treatment indicated that the 5D4-reactive KS is elongated from an O-GalNAc or O-Man linkage. We concluded that sialylated and fucosylated KS, which was thus far reported in cartilage fibromodulin,^{43,48,49} is elicited in microglia with ALS pathogenesis.

GlcNAc6ST1 and KSGal6ST are responsible for the synthesis of KS in the early developing brain.¹² We expected that the up-regulation of sulfotransferases is the cause of the

induction of the 5D4 epitope of KS in SOD1^{G93A} mice. We found that both mRNA and protein levels of GlcNAc6ST1 are up-regulated in SOD1^{G93A} spinal cords at the disease end stage, whereas the KSGal6ST levels are comparable with those of nontransgenic spinal cords. Up-regulation of GlcNAc6ST1 is consistent with the finding that *Chst2* was identified as one of the 40 most differentially expressed genes in SOD1^{G93A} microglia.⁴⁵ These results suggested that GlcNAc6ST1 might be a determinant of the expression of the 5D4 epitope in SOD1^{G93A} spinal cord *in vivo*. The genetic ablation of sulfotransferase in the spinal cord and brain, as shown in SOD1^{G93A}/GlcNAc6ST1^{-/-} and SOD1^{G93A}/KSGal6ST^{-/-} mice, indicates that both GlcNAc6ST1 and KSGal6ST are required for the synthesis of the microglial 5D4 KS epitope elicited in SOD1^{G93A} spinal cord and brainstem. In accordance with this, our transfection experiments revealed that GlcNAc6ST1 and KSGal6ST cooperate in the synthesis of the 5D4 KS epitope in mouse microglia. Cerebral expression of the 5D4 epitope occurs selectively in the brainstem and the white matter of deep cerebellum nucleus, despite both GlcNAc6ST1 and KSGal6ST being expressed in regions of the SOD1^{G93A} brain tested. Although cells that express these sulfotransferases should be identified in the SOD1^{G93A} brain, a plausible explanation for the selective elicitation of the 5D4 epitope could be that there is an additional requirement for the expression of KS glycosyltransferases or core proteins that are potentially modified with the KS epitope in microglia. It was reported that elongation of the carbohydrate backbone of the KS chain is catalyzed by two glycosyltransferases, β 3GnT7 and β 1,4-galactosyltransferase 4.^{50–52} In addition to these enzymes, β 3GnT2 is proposed to be responsible for KS chain elongation.⁵² We found that mRNA of β 3GnT7 is up-regulated in the SOD1^{G93A} spinal cord. It is conceivable that β 3GnT7 is another enzyme required for 5D4-reactive KS synthesis in microglia. This is consistent with the fact that co-transfection of cDNA of β 3GnT7 enhanced generation of the 5D4 epitope in BV-2 and N9 mouse microglia (data not shown). Additional studies will be needed to determine the mechanisms underlying the up-regulation of GlcNAc6ST1 in the ALS model SOD1^{G93A} mice.

Efficient central nervous system delivery of neuroprotective factors for motor neurons was shown to extend life span in SOD1^{G93A} mice.⁵³ Insulin-like growth factor 1 and transforming growth factor- β levels were significantly higher in primary microglia isolated from the SOD1^{G93A}/KSGal6ST^{-/-} spinal cord. Such up-regulation was not seen in primary microglia isolated from SOD1^{G93A}/GlcNAc6ST1^{-/-} mice. These factors may explain, in part, the differences in life span between SOD1^{G93A}/GlcNAc6ST1^{-/-} and SOD1^{G93A}/KSGal6ST^{-/-} mice, despite the elimination of microglial 5D4-KS being detected in both mice. Identification of the molecules bearing the 5D4 epitope in microglia would address these issues and help gain more insight into functional roles of KS in microglia.

Mac2 immunoreactivity is detected in activated and proliferating microglia associated with chronic or acute neuronal deficits, but absent in quiescent microglia.⁵⁴ The 5D4-reactive KS is expressed in Mac2⁺ microglia in the SOD1^{G93A} spinal cord and brainstem. In the spinal cord and brainstem, lower motor neurons and upper motor neuronal tracts are present. Several studies have indicated that the neuropathology in SOD1^{G93A} mouse is confined largely to the degeneration of spinal and bulbar lower motor neurons but not in corticospinal upper motor neurons.^{55–57} The 5D4-KS was not detected in the SOD1^{G93A} cerebral cortex. In accordance with the results in SOD1^{G93A} mice, we found that expression of the 5D4-KS was apparently up-regulated in postmortem spinal cords of ALS patients. Thus, 5D4-KS expression is considered to occur in parallel to degeneration of the lower motor neurons in ALS.

Evidence has found that a subset of activated microglia have a potential role in neuronal functions by interacting with neurons and releasing neurotoxic or neurotrophic molecules and inflammatory cytokines.⁵⁸ Our immunoelectron microscopy observations suggested that the 5D4-reactive KS might also be involved in microglial surveying and sensing the local environment by contacting other cells. 5D4 immunoreactivity could be useful to characterize a microglial state with activation in central nervous tissues.

Increasing evidence points to the involvement of the immune system in the pathogenesis of ALS.⁵⁹ The presence of T lymphocytes in the spinal cord of both ALS patients and ALS animal models was reported.^{60–62} The 5D4 epitope is expressed in a subpopulation of microglia that are CD86⁺.³⁴ It is assumed that a subset of the 5D4-expressing microglia may serve as proliferating antigen-presenting cells that regulate a neurodegeneration-induced inflammatory environment in the SOD1^{G93A} spinal cord by providing costimulatory signals to infiltrating T lymphocytes. The 5D4-reactive KS expressed on the cell surface of activated/proliferating microglia could coordinate this intercellular interaction.⁶³ CD4⁺ T lymphocytes infiltrate into the lumbar spinal cord before the symptomatic stage and are required for the induction of alternatively activated microglia, which augment a neuroprotective environment in the SOD1^{G93A} spinal cord.^{60,61} Another possible contribution of the sulfotransferase might be elaboration of selectin ligand mucins,⁴¹ being involved in the extravasation of T lymphocytes into neurodegenerating spinal cord and their modulation of the microglial trophic and cytotoxic balance. A decreased level of IL-4 in the SOD1^{G93A}/GlcNAc6ST1^{-/-} spinal cord at pre-symptomatic stages³⁴ could perhaps be due to reduced levels of infiltrated CD4⁺ T lymphocytes. These possibilities should be addressed in future study.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.07.016>.

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