Keratan sulfate (KS) is a glycosaminoglycan composed of repeating disaccharide units with sulfate residues at the C6 positions of galactose and N-acetylgalactosamine (GlcNAc). The N-acetylgalactosamine 6-O-sulfotransferase(s) (GlcNAc6ST) involved in the synthesis of KS in the central nervous system (CNS) has long been unidentified. Here, we report that a deficiency of GlcNAc6ST-1 leads to loss of 5D4-reactive brain keratan sulfate (KSPG), was abolished in the brains of GlcNAc6ST-1–/– mice. More recently, taking advantage of that condition of the deficient mice, we found that KS plays an indispensable role in glial scar formation after cortical stab injury in mice. During the development of mice deficient in GlcNAc6ST-1, KS expression in the brain was barely detectable with the KS-specific antibody 5D4. The reactivity of 5D4 antibody with protein tyrosine phosphatase ζ (PTPζ), a KS proteoglycan (KSPG), was abolished in the deficient mice. In adults, brain injury induced 5D4-reactive KS synthesis in the wounded area in wild-type (WT) mice but not in the deficient mice. Glial scar is formed via the accumulation of reactive astrocytes and is a major obstacle to axonal regeneration of injured neurons. Reactive astrocytes appeared to similar extents in the two genotypes, but they accumulated in the wounded area to a lesser extent in the deficient mice. Consequently, the deficient mice exhibited a marked reduction of scarring and enhanced neuronal regeneration after brain injury. These findings highlight the indispensable role of GlcNAc6ST-1 in brain KS biosynthesis and glial scar formation after brain injury.

Key words: axon regeneration/glial scar/keratan sulfate/N-acetylgalactosamine 6-O-sulfotransferase/reactive astrocytes

Introduction

Keratan sulfate (KS) is a glycosaminoglycan, which is formed through the elongation of N- or O-glycans covalently attached to scaffold proteins. KS-bearing proteins are known as KS proteoglycans (KSPGs) and are found in the extracellular matrix and on the cell surface (Klene and Schachner, 2004). KS is composed of repeating disaccharide units of galactose and N-acetylgalactosamine (GlcNAc), with sulfate groups at the C6 positions of galactose and GlcNAc. The sulfation modification of GlcNAc residues is mediated by GlcNAc6ST (C-GlcNAc6ST/GlcNAc6ST-5/CHST6) (Fukuda et al., 2001). Human corneal GlcNAc6ST (C-GlcNAc6ST) is critical for KS synthesis in the cornea (Akama et al., 2000, 2001), which is a tissue that contains abundant KSPGs (Nilsson et al., 1983). A previous study showed that sulfation on the C6 positions of GlcNAc residues is necessary for KS chain elongation (Akama et al., 2001).

KS is also expressed in central nervous tissues (Miller et al., 1997). Interestingly, KS synthesis is up-regulated in the lesions on central nervous system (CNS) injury (Jones and Tuszynski, 2002). The adult mammalian CNS does not spontaneously regenerate after injury. The failure of the regeneration of injured axons is widely acknowledged to be due to a combination of factors, including the emergence of a molecular barrier because of the up-regulation of growth-inhibiting molecules such as inhibitors within myelin and chondroitin sulfate proteoglycans (CSPGs), and the formation of a physical barrier by reactive astrocytes (“glial scar”) (Horner and Gage, 2000; Filbin, 2003; McGee and Strittmatter, 2003; Schnaar, 2004). KS is also expressed in central nervous tissues (Miller et al., 1997). Interestingly, KS synthesis is up-regulated in the lesions on central nervous system (CNS) injury (Jones and Tuszynski, 2002). The adult mammalian CNS does not spontaneously regenerate after injury. The failure of the regeneration of injured axons is widely acknowledged to be due to a combination of factors, including the emergence of a molecular barrier because of the up-regulation of growth-inhibiting molecules such as inhibitors within myelin and chondroitin sulfate proteoglycans (CSPGs), and the formation of a physical barrier by reactive astrocytes (“glial scar”) (Horner and Gage, 2000; Filbin, 2003; McGee and Strittmatter, 2003; Schnaar, 2003; Silver and Miller, 2004). However, molecular mechanisms underlying the glial scar formation have not been well understood.

GlcNAc6ST-1 mRNA is expressed in various tissues including the brain (Uchimura et al., 1998; Fan et al., 1999). Here, we demonstrate that KS, which is recognized by the KS-specific antibody 5D4, is abrogated in the brains of GlcNAc6ST-1-deficient (GlcNAc6ST-1–/–) mice. Moreover, taking advantage of that condition of the deficient mice, we found that KS plays an indispensable role in glial scar formation after brain injury.

Results

Loss of 5D4 and EFG11 reactivity in GlcNAc6ST-1–/– mice during development

We first examined KS expression in the developing brain, because we had found that GlcNAc6ST-1 mRNA is expressed in the thalamus of mouse embryos (Fan et al., 1999). The 5D4 antibody specifically recognizes KS (Scudder et al., 1986). 5D4-reactive KS expression was detected in the embryonic thalamus and cerebral cortex in wild-type (WT) mice but not in GlcNAc6ST-1–/– mice (Figure 1A and B). By contrast, 5D4-reactive KS was expressed in the cartilage of developing vertebrae in a similar manner in the
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two genotypes (Figure 1C). On postnatal day 1, 5D4-reactive KS was expressed in several nuclei in the thalamus and the projecting route from the thalamus to the cortex in WT mice, this being consistent with a previous report describing 5D4-reactive KS expression in rat brain (Miller et al., 1997) (Figure 1D). This expression had completely disappeared in GlcNAc6ST-1–/– mice (Figure 1D).

KS expression in WT mouse brain was further confirmed by means of western blot analysis with 5D4. Broad bands corresponding to between 250 and 150 kDa, and higher than 250 kDa, appeared on postnatal days 1 and 8 and had diminished slightly on day 15 (Figure 2A). A band higher than 250 kDa is indicated by asterisks shown in Figure 2E. Digestion of WT brain samples with keratanase I or keratanase II significantly decreased the 5D4 reactivity observed on western blot, indicating the specificity of this antibody (Figure 2B). Both 5D4 and EFG11, which recognizes poorly sulfated KS, detected the smear bands for WT mice brain but not for GlcNAc6ST-1–/– mice (Figure 2C and D).

Protein tyrosine phosphatase ζ (PTPζ)/phosphacan is known to be a KS-bearing proteoglycan in the nervous system (Maeda et al., 1995). The 6B4 antibody recognizes this proteoglycan and precipitated similar amounts of PTPζ in the two genotypes (Figure 2E, left). However, only the precipitate obtained with 6B4 antibody from WT mice, that is, not that from GlcNAc6ST-1–/– mice, contained 5D4-reactive KS (Figure 2E, left). Similarly, the 5D4 antibody only precipitated PTPζ in WT mice (Figure 2E, right). These data collectively indicate that the loss of GlcNAc6ST-1 leads to the abrogation of 5D4-reactive KS in the embryonic and neonatal brains of mice.

Fig. 1. 5D4-positive signals are absent in the brain, but not the vertebrae, in GlcNAc6ST-1–/– mice during development. (A) Brain specimens obtained from embryonic day 15.5 mice were stained with 5D4 antibody. The results for WT (+/+) and GlcNAc6ST-1–/– (–/–) mice are shown. Dark field photos show KS expression in red color. Bright field photos show hematoxylin staining. DT, dorsal thalamus. Bars: 250 μm. (B) Brain specimens obtained from embryonic day 15.5 mice were stained with 5D4 antibody. Dark field photos show KS expression in red color. Bright field photos show hematoxylin staining. CP, cortical plate; DT, dorsal thalamus; IZ, intermediate zone; SP, subplate. (C) Vertebra specimens from embryonic day 15.5 mice were stained with 5D4 antibody. Bright field photos show hematoxylin and eosin staining. (D) Brain specimens on postnatal day 1 were also stained with 5D4 antibody. Bright field photos show hematoxylin and eosin staining. IC, internal capsule; LH, lateral hypothalamic area; VPL, ventral posterolateral thalamic nucleus; VPM, ventral posteromedial thalamic nucleus. Bars: 250 μm.

Loss of 5D4-staining signals in GlcNAc6ST-1–/– mice after brain injury

Despite such a specific expression profile of KS during development, GlcNAc6ST-1–/– mice did not show any apparent abnormalities of the CNS. It is known that KS
expression is substantially induced by injury in a model of rat spinal cord injury, as revealed by 5D4 antibody immunostaining (Jones and Tuszynski, 2002). To study the biological function of KS in CNS injury, we performed an assay in which a stab wound was made to the cerebral cortex. 5D4-reactive KS expression became apparent in WT mice 4 days after injury, reached the maximum level at 7 days, and then gradually decreased (Figure 3A). However, the induction of 5D4-reactive KS expression was not observed in GlcNAc6ST-1–/– mice (Figure 3A). Chondroitin sulfate (CS) expression was induced to similar extents in the two genotypes (Figure 3B). Consistently, NG2, a CSPG, was induced in both genotypes (Figure 3C) (Jones et al., 2002).

Supporting the results of 5D4-reactive KS expression induction in WT mice, GlcNAc6ST-1 expression was strongly up-regulated by injury, as revealed by reverse transcription-polymerase chain reaction (RT–PCR) using the cDNAs as templates. Six WT mice (three for injured and three for control samples) were used.

Reduction of glial scar formation in GlcNAc6ST-1–/– mice after brain injury
Glia fibrillary acidic protein (GFAP) is a marker for reactive astrocytes. In WT mice, reactive astrocytes appeared around the wounded area 2 days after injury and had accumulated around the lesion by 7 days (Figure 4A). The accumulation was most prominent at 10 days and then gradually decreased (Figure 4A). However, the astrocyte accumulation was strikingly reduced in GlcNAc6ST-1–/– mice, although reactive astrocytes appeared around the wounded area to an extent similar to that in WT mice at
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2 days (Figure 4A). Quantitative analysis revealed that the accumulation of reactive astrocytes around the injured lesion was significantly suppressed in GlcNAc6ST-1−/− mice (Figure 4B, methods are described in Materials and Methods). Interestingly, the 5D4-reactive KS expression area was more restricted than the reactive astrocyte accumulation area 7 days after injury in WT mice (Figure 4C). Taking the temporal profiles of 5D4-reactive KS expression (peak at 7 days after injury; Figure 3A) and reactive astrocyte accumulation (peak at 10 days; Figure 4A) into account, the results indicate that reactive astrocytes migrated to the 5D4-reactive KS-expressing area, that is, the lesion core, in WT mice. Figure 4C also demonstrates that cells expressing 5D4-reactive KS and GFAP did not overlap. This is consistent with a previous report that 5D4-reactive KS is produced by invading macrophages, microglia, and oligodendrocyte precursors but not by astrocytes in spinal cord injury (Jones and Tuszynski, 2002).

Collagen IV appears at late stages of glial scarring (Liesi and Kauppila, 2002) and is associated with an activity that inhibits axonal regeneration (Stichel et al., 1999). Collagen IV expression in the wounded area became apparent 14 days after injury in WT mice, whereas it was negligible in the wounded area in GlcNAc6ST-1−/− mice (Figure 5), this being consistent with the very low accumulation of reactive astrocytes in these mice (Figure 4A). These results support the conclusion that glial scarring was markedly reduced in GlcNAc6ST-1−/− mice.

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**Fig. 4.** Reactive astrocyte accumulation following a stab wound is strikingly reduced in GlcNAc6ST-1−/− mice. (A) Anti-GFAP antibody was used for this immunofluorescence staining. Days after a stab wound are shown on the left side of each row. At least three mice were examined for each time point and showed similar results. Representative photos are shown. Bars: 250 μm. (B) The accumulation of reactive astrocytes at 7 and 10 days after injury is presented as a ratio of GFAP-positive cells in the lesion core versus those in the whole area of obtained images. The quantification method is described in Materials and Methods. **p < 0.001; *p < 0.01. (C) Double staining using anti-GFAP and anti-KS 5D4 was performed. Three mice were examined for each time point and showed similar results. Representative photos are shown. Bars: 250 μm.

**Fig. 5.** Collagen IV expression following a stab wound is strikingly reduced in GlcNAc6ST-1−/− mice. (A) Collagen IV staining of brain samples at 14 days after injury. Three mice were examined and showed similar results. Representative photos are shown. Bars: 250 μm. (B) Quantification of the collagen IV-positive area at 14 days after injury was performed as described in Materials and Methods. **p < 0.001.
Enhancement of axonal regeneration in GlcNAc6ST-1−/− mice after brain injury

Coinciding with the abolished 5D4-reactive KS expression and poor glial scar formation, axonal regeneration was pronounced in GlcNAc6ST-1−/− mice. The SMI312 antibody can detect phosphonеurofilaments, particularly those of regenerating axons of injured neurons (King et al., 2001). SMI312-positive signals were much more conspicuous at 4 and 7 days after injury in GlcNAc6ST-1−/− mice than in WT mice (Figure 6A and B). Another marker for regenerating axons, anti-growth-associated protein-43 (GAP-43) (King et al., 2001; Bradbury et al., 2002), also demonstrated enhanced axonal regeneration in GlcNAc6ST-1−/− mice (Figure 6C).

Discussion

GlcNAc6ST-1, together with GlcNAc6ST-2, has been regarded as an enzyme required for the synthesis of an L-selectin determinant, sialyl 6-sulfo Le\(^\alpha\), on high endothelial venules in lymph nodes (Hemmerich et al., 2001; Uchimura et al., 2004). More recently, we have demonstrated that mice deficient in both GlcNAc6ST-1 and GlcNAc6ST-2 genes exhibit the elimination of sialyl 6-sulfo Le\(^\alpha\) on high endothelial venules and striking reduction of lymphocyte homing (Kawashima et al., 2005; Uchimura et al., 2005). In contrast, the GlcNAc6ST(s) involved in the synthesis of KS in CNS has long been unidentified. Our previous studies showed that GlcNAc6ST-1 transcripts are expressed in the neocortex and dorsal thalamus of mouse embryos (Fan et al., 1999). These results strongly support that the loss of 5D4-reactive KS in the brain, including the neocortex and dorsal thalamus, of GlcNAc6ST-1−/− mouse embryos is attributable to deficiency of C-6 sulfation of GlcNAc residues in KS mediated by GlcNAc6ST-1. The up-regulation of GlcNAc6ST-1 mRNA in the injured brains of adult mice revealed by RT–PCR correlates with highly induced synthesis of 5D4-reactive KS in the lesions on injury in WT mice. This induction was not seen in GlcNAc6ST-1−/− mice after injury, indicating that GlcNAc6ST-1 is the enzyme that elaborates 5D4-reactive KS in the injured brains of adult mice. This is the first evidence that GlcNAc6ST-1 is an enzyme that is involved in GlcNAc-6-sulfation of KS in the mouse CNS. GlcNAc is also one of the disaccharides that make up heparan sulfate and hyaluronic acid. We previously showed that GlcNAc6ST-1 does not exhibit any activity toward completely desulfated \(N\)-resulfated heparin (Uchimura et al., 1998). In addition, the existence of sulfated hyaluronic acid has not been reported so far. Thus, we conclude that the loss of GlcNAc6ST-1 only affects the synthesis of KS among glycosaminoglycans.

To address a possible role of poly-\(N\)-acetyllactosamine in the recovery after brain injury, we performed a lectin blot analysis using \(Erythrina cristagalli\) lectin (ECA). ECA recognizes \(N\)-acetyllactosamine and poly-\(N\)-acetyllactosamine, the latter signal becoming faint after endo-\(\beta\)-galactosidase digestion. We found that ECA-reactive signals became stronger after brain injury to similar extents in the two genotypes, that is, WT and GlcNAc6ST-1−/− mice (data not shown). However, those signals were only slightly weaker after endo-\(\beta\)-galactosidase digestion (data not shown). Therefore, it is not likely that poly-\(N\)-acetyllactosamine plays a major role in the recovery after brain injury in

Fig. 6. Neuronal regeneration is enhanced in GlcNAc6ST-1−/− mice. To monitor neuronal regeneration, SMI132 (A) and GAP-43 (C) antibodies were used. SMI132-positive fibers at 7 days after injury (B) was also quantified as described in Materials and Methods. The photos in the second row in (A) are higher magnifications of areas (squares) depicted than those in the first row. Data of 7 days after injury in (A) and (C) are serial sections from the same mouse. Days after a stab wound are shown on the left side of each row. At least three mice were examined for each time point and showed similar results. Representative photos are shown. Bars represent 250 \(\mu\)m, except for the second row in (A), in which bars represent 62.5 \(\mu\)m. ** \(p < 0.001\).
GlcNAc6ST-1−/− mice. This idea is supported by literatures, showing that synthesis of KS is independent of that of poly-N-acetyllactosamine (Akama et al., 1998, 2001; Lee et al., 2000; Uchimura et al., 2002; Seko et al., 2003; Seko and Yamashita, 2004). Thus, in the KS repeating units of Galβ1-4GlcNAcβ1-3, GlcNAc is always 6-sulfated and Gal is occasionally sulfated. The sequence of KS biosynthesis is N-acetylgalcosaminylolation, 6-sulfation of a GlcNAc residue exposed at the non-reducing end, and galactosylation.

A stab wound induced the appearance of reactive astrocytes in GlcNAc6ST-1−/− mice, which was comparable with that in WT mice. Surprisingly, however, reactive astrocyte accumulation in the lesion core and subsequent collagen IV expression were markedly reduced in GlcNAc6ST-1−/− mice. These in vivo results suggest that 5D4-reactive KS itself or attractive factors recruited to 5D4-reactive KS in the lesion core may promote astrocyte accumulation. Although the mechanism of KS-mediated astrocyte migration remains to be verified, our data highlight an indispensable function of KS in the migration of reactive astrocytes and glial scar formation.

KSPGs are expressed in the roof plate of the developing spinal cord, being involved in barrier formation during ontogenesis (Snow et al., 1990; Cole and McCabe, 1991). Moreover, treatment with keratanase but not with chondroitinase ABC leads to enhanced outgrowth and the regeneration of transected mossy fiber in rat hippocampal slice cultures (Butler et al., 2004). These results suggest a non-permissive role of KS as to neuronal regeneration. This is supported by our finding that the enhancement of axonal regeneration started in GlcNAc6ST-1−/− mice at 4 days after injury when 5D4-reactive KS expression was up-regulated in WT mice, this timing being earlier than that of apparent accumulation of astrocytes in WT mice (7 days after injury). It is also known that KS inhibits axonal outgrowth in vitro (Dou and Levine, 1995). Therefore, our results further support the importance of KS’s inhibitory activity as to axonal growth.

The two roles (as to glial scar formation and neuronal regeneration) of KS addressed above could be more clearly understood by comparing KS with CS. One of the most important findings in the present study is that CS expression was induced to similar extents in both WT and GlcNAc6ST-1−/− mice, which was comparable with that in WT mice. Surprisingly, however, reactive astrocyte accumulation in the lesion core and subsequent collagen IV expression were markedly reduced in GlcNAc6ST-1−/− mice. These in vivo results suggest that 5D4-reactive KS itself or attractive factors recruited to 5D4-reactive KS in the lesion core may promote astrocyte accumulation. Although the mechanism of KS-mediated astrocyte migration remains to be verified, our data highlight an indispensable function of KS in the migration of reactive astrocytes and glial scar formation.

Materials and methods

Mice

GlcNAc6ST-1−/− mice were produced using D3 embryonic stem cells and an ordinary gene targeting technology as previously described (Uchimura et al., 2004). GlcNAc6ST-1−/− mice obtained after backcrossing with C57BL/6 for more than seven generations were interbred, and the litters obtained were used for the brain injury experiments. These mice were maintained in the animal facilities of Nagoya University. All experiments were performed in accordance with protocols approved by the institutional animal committee.

Reagents

Anti-KS 5D4 antibody and biotin-conjugated one were purchased from Seikagaku (Tokyo, Japan). EFG11 was from Chemicon, USA. Anti-CS CS-56 antibody, Cy3-conjugated anti-GFAP antibody, and fluoresceinisothiocyanate (FITC)-conjugated anti-mouse IgG were from Sigma (Kanagawa, Japan); anti-phosphondurofilament SM132 from Sternberger Monoclonals (Lutherville, MD); anti-GAP-43 and anti-NF2 from Chemicon; and anti-type IV collagen from LSL (Tokyo, Japan). Cy3- or Cy2-conjugated streptavidin and Cy3-conjugated anti-mouse IgM were from Jackson ImmunoResearch (West Grove, PA); Cy3-conjugated anti-rabbit IgG from Zymed (Tokyo, Japan); FITC-conjugated anti-rabbit IgG from Cappel (Irvine, CA); and anti-β actin antibody from Sigma. FluorSave was obtained from Calbiochem, (Tokyo, Japan).

Immunohistochemistry

Tissues were cut into 5-μm sections with a cryostat and mounted on glass slides. Sections were fixed with cold acetone
placed in a stereotaxic frame. Cortical injury was induced in Six-week-old male C57BL6J mice were anesthetized and with chondroitinase ABC (Seikagaku; 1 mU/μg protein, 0.1 M Tris–HCl, pH 7.3) or keratanase II (Seikagaku; 1 mU/μg protein, 0.1 M Tris–HCl, pH 7.3) for 2 h at 37 °C. The cerebral cortex and thalamus were dissected from the brains of P1, P8, and P15 mouse and homogenized in PBS including 1% Triton X-100 and protease inhibitors solution (Sigma). Samples of the supernatant fraction were collected after centrifuging at 10,000 g for 30 min and were separated by electrophoresis on 6% SDS–PAGE. Proteins were then blotted onto nitrocellulose membranes. Blots were blocked with 5% fat-free dry milk in PBS containing 0.3% Triton X-100 for 60 min and incubated overnight at 4°C with the primary antibody anti-KS 5D4 (1 μg/mL) or EFG11 (1/1000) in PBS containing 0.3% Triton X-100, washed, and then incubated with second antibody horse-radish peroxidase-conjugated goat anti-mouse IgG (1/1000) at 4°C. Bound antibodies were visualized with an ECL western blotting detection kit (Amersham Biosciences, Foster City, CA). The membrane was reprobed with anti-β-actin antibody after blocking with 5% fat-free dry milk in PBS containing 0.3% Triton X-100 solution once more.

For immunoprecipitation, 100 μg of proteins from the thalamus (extracted as above) was digested with chondroitinase ABC and then mixed with anti-KS 5D4 (5 μg) or anti-PTPζ antibody (2 μg) (6B4, a generous gift from N. Maeda and M. Noda) for 2 h at 4°C. The immune complex was precipitated with 30 μL of a 50% (v/v) suspension of protein A-sepharose (Amersham Biosciences) for 1 h at 4°C and washed three times with PBS containing 0.3% Triton X-100. The immunocomplexes bound to the protein A beads were isolated by centrifugation and were subjected to immunoblots.

Enzymatic treatment
Protein samples from the thalamus (extracted as above) were treated with keratanase I (Seikagaku; 5 μU/100 μg of protein, 0.1 M Tris–HCl, pH 7.3) or keratanase II (Seikagaku; 1 μU/μg of protein, 0.1 M sodium acetate, pH 6.5) for 4 h at 37°C. The immunoprecipitation samples were treated with chondroitinase ABC (Seikagaku; 1 μU/μg protein, 0.1 M Tris–acetate, pH 7.3) for 2 h at 37°C.

Controlled cortical stab injury and knife-cut injury
Six-week-old male C57BL6J mice were anesthetized and placed in a stereotaxic frame. Cortical injury was induced in the left parietal cortex. The cortical coordinates were 1.2 mm anterior and 1.0 mm lateral as to the bregma and a depth of 1 mm from the dura. A needle (1 mm in diameter) was lowered into the brain through a small burr hole drilled in the skull. For a knife-cut model, a knife cut (2.5 mm depth and 6.0 mm length) 1.0 mm lateral as to the bregma was made, and the lesions and the corresponding regions from normal mice were isolated 7 days after injury.

RT–PCR
A set of a forward primer (5’-AAGCCTACAGGTGTGTC GAA-3’) and a reverse primer (5’-CAGGACTGTTAAC CGCCTCA-3’) was used for RT–PCR for GlcNAc6ST-1 expression, and a set of a forward primer (5’-GGTTGAG GTGGAGTCAACG-3’) and a reverse primer (5’- CAAAGTGTACGATGACC-3’) were used for RT–PCR for GAPDH expression. SuperScript III reverse transcriptase (Invitrogen, Tokyo, Japan) was used to synthesize cDNA.

Morphometry
The midpoint of a lesion was determined by hematoxylin and eosin staining of several sections from serial 5-μm sections. To count reactive astrocytes, areas of specimens were traced, and 640 × 2200-μm² counting frames were selected by a computer-driven microscope stage (MetaMorph Offline version 6.3r2, Molecular Devices Corporation, Sunnyvale, CA) for counting of astrocytes around the lesions, and 216 × 100 μm² counting frames were selected for astrocytes accumulated in the lesion core. The accumulated astrocytes/total reactive astrocytes ratio was determined. The extents of extracellular matrix (ECM) and axonal outgrowth of the wound area were assessed by counting signals visualized on staining with anti-type IV collagen and SMI312 antibodies, respectively, for 640 × 2200-μm² counting frames around a lesion with the aid of computerized image analysis with the same soft program. Data were collected for at least three mice with each genotype in each experiment.

Statistical analysis
All data are presented as means ± SEM. Statistical analysis was performed with Student’s t-test, and p < 0.05 was considered as being significant.

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Conflict of interest statement
None declared.
Abbreviations

BSA, bovine serum albumin; CNS, central nervous system; CS, chondroitin sulfates; ECA, Erythrina cristagalli lectin; ECM, extracellular matrix; FITC, fluoresceinisothiocyanate; GAP, growth-associated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GlcNAc6ST, N-acetylglucosamine 6-O-sulfotransferase; KS, keratan sulfate; PBS, phosphate-buffered saline; PG, proteoglycan; PTP, protein tyrosine phosphatase; RT–PCR, reverse transcription–polymerase chain reaction; WT, wild type.

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