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Keratan sulfate expression is associated with activation of a subpopulation of microglia/macrophages in Wallerian degeneration

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HIGHLIGHTS

- A hemilateral section of the cervical cord induced KS expression at the lumbar.
- The KS signal was merged with a subpopulation of microglia/macrophages.
- These cells also expressed CD68 and CD86, but not CD206 or arginase 1.
- These microglia/macrophages were in an activated state probably polarized to M1.

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ABSTRACT

Wallerian degeneration is a fundamental process of axonal degeneration distal to the injury site. Although axonal degeneration itself is accomplished in a few days, the subsequent process of removing debris, including myelin debris, in the central nervous system takes more time. Since this debris is a potent inhibitor of axonal regeneration, the removal process is critical for functional recovery after neuronal injuries. Although it is known that microglia/macrophages are involved in this process, the underlying mechanisms are not fully understood. Here, we found that keratan sulfate (KS) expression was induced far from the injury site after spinal cord injury. A hemilateral section of the spinal cord at the third cervical level induced KS expression in a restricted area of the ipsilateral column at the first lumbar level 1 week after injury. This localized KS expression lasted for at least 1 month after injury. The KS signal was merged with a portion of Iba1-positive cells, suggesting that a subpopulation of microglia/macrophages expressed KS. KS-positive cells expressed CD68 and CD86, but not CD206 or arginase 1, suggesting that these microglia/macrophages were in an activated state probably polarized to M1. Our study has explored for the first time the relation between KS expression and activation of microglia/macrophages in Wallerian degeneration.

1. Introduction

Wallerian degeneration is defined as degeneration of an axon distal to the injury site. It accompanies glial activation to remove debris of myelin and other components [14–16]. The removal of myelin debris is an important step for functional recovery after neuronal injuries, since this debris works as a strong inhibitor of axonal regeneration/sprouting. While axonal degeneration is complete at 3 days after injury, the removal of myelin debris takes several months [1,4]. Activated microglia/macrophages are detected after spinal cord injury in the dorsal ascending tract rostral to the lesion and in the corticospinal tract caudal to the lesion (Wang et al. [16]). These activated microglia/macrophages are thought to be involved in myelin debris removal [2,10,17].

We and others found that keratan sulfate expression is induced at the site of neural injury [6–8,18]. While chondroitin sulfate (CS) is known as a potent inhibitor of axonal regeneration/sprouting, we demonstrated that KS is another inhibitor and that its removal promotes functional recovery after spinal cord injury [6,7]. KS is expressed by not only reactive astrocytes but also activated microglia/macrophages at the injury site [6,7]. Although it is likely that KS produced by astrocytes is involved in the inhibition of axonal regeneration/sprouting, the biological significance of KS expressed in microglia/macrophages has not been well explored.

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Here, we demonstrate that KS expression is closely associated with Wallerian degeneration and is linked to the activation of a subpopulation of microglia/macrophages.

2. Materials and methods

2.1. Animal subjects and surgery

Adult female C57BL/6J mice (8 weeks old) were housed under a 12 h light-dark cycle in standard cages with free access to food and water *ad libitum*. The mice were anesthetized with an intraperitoneal injection of somnopentyl (60 mg/kg, Kyoritsu Seiyaku). After laminectomy at the third cervical spinal lamina, we exposed the dorsal surface of the dura mater. A right spinal cord lateral hemisection was performed at spinal C3 with a fine scalpel. The muscles and skin layers were then sutured. In sham-operated mice, the skin wound was closed without a spinal cord lateral hemisection.

As postoperative care, the bladder was compressed by manual abdominal pressure twice per day until bladder function was restored, and prophylactic antibiotic treatment 1.0 ml of Bactramin (Roche) in 500 ml of acidified water was maintained for 1 week.

2.2. Anterograde labeling of the CST

One day after spinal cord lateral hemisection, a tracer was injected into the bilateral primary motor cortex in order to visualize the CST. The spinal cord lateral hemisection mice were anesthetized as described above and placed on a stereotaxic device. The skull overlying both sides of the primary motor cortex was removed. Biotinylated dextran amine (BDA) (molecular weight, 10,000; dilution, 10% in phosphate-buffered saline (PBS); Invitrogen), an anterograde tracer, was injected at six sites (0.5 μl/site) located in the hindlimb area of the motor cortex. Two weeks after the BDA injection, the mice were sacrificed and the spinal cord was removed.

2.3. Quantitative RT-PCR

The total RNA was extracted from the mouse right lumbar spinal cord after right lateral hemisection using an RNeasy Lipid tissue Mini Kit (Qiagen) according to the manufacturer's instructions. The cDNA was prepared from 1 μg of total RNA by using a Transcriptor First Strand cDNA Synthesis Kit (Roche) following the standard protocols. Quantitative PCR was practiced on Mx3000P or Mx3005P (Agilent Technologies) using SYBR Green (Agilent Technologies). The samples were subjected to 40 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min, after being held at 50 °C for 2 min and 95 °C for 10 min. Relative expression was calculated using the $2-(\text{Cr experimental sample}-\text{Cr internal control sample (GAPDH)})$ according to the protocol. Real-time PCR was performed using a Light Cycler 480 Real-Time System. The sequences of the primers used for the quantitative RT-PCR of M1 and M2 markers are listed in Table 1.

2.4. Immunohistochemistry

Mice were anesthetized with somnopenthyl and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (Wako). Isolated spinal cords were fixed with 4% PFA overnight at 4 °C, and cryopreserved by 20% sucrose in 0.1 M phosphate buffer at 4 °C during the subsequent night. The tissue samples were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek) and quickly frozen by liquid nitrogen. Frozen tissues were cut into 20 μm sections on a cryostat (CM3050; Leica Instruments) and collected on MAS-coated glass slides (Superfrost; Matsunami Glass).

The sections were washed in PBS. After blocking with 3% bovine serum albumin (BSA) in PBS containing 0.3% Triton X-100 for 30 min at room temperature, the sections were then incubated at 4 °C overnight with the following primary antibodies: mouse anti-KS (1:400, clone 5D4; Seikagaku), rabbit anti-Iba1 (1:500; Wako), rabbit anti-GFAP (1:1000; Sigma), anti-Olig2 (1:50; Santa Cruz Biotechnology), rat anti-CD68 (1:200; AbD Serotec), rat anti-CD86 (1:200; BD Biosciences), rat anti-CD206 (1:200; AbD Serotec), and gout anti-arginase 1 (1:50; Abcam).

After rinsing in PBS, the sections were incubated for 60 min at room temperature with the secondary antibodies: Alexa Fluor 488 conjugated donkey anti-mouse IgG (Invitrogen), Alexa Fluor 488 conjugated donkey anti-goat IgG (Invitrogen), Alexa Fluor 594 conjugated donkey anti-mouse IgG (Invitrogen), Alexa Fluor 594 conjugated donkey anti-rat IgG (Invitrogen), and Alexa Fluor 647 conjugated donkey anti-rabbit IgG (Invitrogen). The sections were rinsed in PBS, mounted with FluorSave (Dako), and observed by a BZ-9000 microscope (Keyence) fitted with the appropriate filters.

2.5. Statistical analysis

An unpaired Student's two-tailed *t*-test was used to count the cells in the time courses of KS- and Iba1-positive cells. Two-way ANOVA was used to analyze the mRNA expression of M1 and M2 markers in the time courses. In all statistical analyses, values of $p < 0.05$ were considered to indicate significance. The statistical analysis was performed using SPSS Statistics 20 (SPSS) software. The investigators performing statistical analyses were blinded to the group assignments in all procedures.

3. Results

3.1. Keratan sulfate is expressed far from the injury site

A left lateral hemisection of the spinal cord was performed at the third cervical level. The cortico-spinal tract tracing with BDA revealed that the left injured CST axon was transected at the injury site, while the right uninjured CST axon descended to caudal (Fig. 1A). The KS-specific antibody 5D4 detected KS expression broadly in the ipsilateral spinal cord at the fifth cervical level 1 week after injury (Fig. 1B, C5). KS expression became restricted

Table 1
The primer sequence using quantitative RT-PCR of M1 and M2 microglial makers.

M1 microglial makers (5′ → 3′)			M2 microglial makers (5′ → 3′)		
CD68	Forward	CCACAGGCAGCACAGTGGACA	CD206	Forward	TCAGCTATTGGACGCGAGGCA
	Reverse	TCCACAGCAGAAGCTTTGGCCC		Reverse	TCCGGGTTGCAAGTTGCCGT
IL-1β	Forward	CCTGCAGCTGGAGAGTGTGGAT	Arginase1	Forward	TTAGGCCAAGGTGCTTGCTGCC
	Reverse	TGTGCTCTGCTTGTGAGGTGCT		Reverse	TACCATGGCCCTGAGGAGGTTC
			IL-4	Forward	TGGGTCTCAACCCCCAGCTAGT
				Reverse	TGCATGGCGTCCCTTCTCCTGT
			IL-10	Forward	GGCAGAGAACCATGGCCCAGAA
				Reverse	AATCGATGACAGCGCCTCAGCC