主論文の要旨

Microglial keratan sulfate epitope elicited in central nervous tissues of transgenic model mice and patients of ALS

（筋萎縮性側索硬化症患者およびモデルマウスの中枢神経組織ミクログリアで発現誘導されるケラタン硫酸糖鎖）

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Summary

Glycan structures in a normal state often differ from those in diseases. Keratan sulfate (KS) is a sulfated glycan that comprises repeating disaccharides of galactose (Gal) and *N*-acetylglucosamine (GlcNAc). KS is known to inhibit neuronal regeneration/sprouting after injury in the central nervous tissues. Roles of KS in neurodegenerative disease are unknown. Monoclonal antibody 5D4 recognizes KS structures that contain Gal and GlcNAc when both are C6-sulfated. Amyotrophic lateral sclerosis (ALS) is a motor neuron-degenerative disease. Here, biochemical analyses revealed that the 5D4-reactive KS is induced in the central nervous tissues 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/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 has thus far been 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 Gal-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 the both Gal-6- and GlcNAc-6-sulfated KS elicited in the spinal cord and brainstem are associated with the degeneration of spinal/bulbar lower motor neurons in ALS pathology and may play a role in disease progression via microglial activation and proliferation.

Materials and Methods

Animals

C57BL/6J, B6.Cg-Tg (SOD1*G93A) 1Gur/J (SOD1G93A), $SOD1^{G93A}$/$GlcNAc6ST1^{-/-}$ and $SOD1^{G93A}$/$KSGal6ST^{-/-}$ mice were used for the experiments.

Human postmortem spinal cord tissues

The diagnosis of ALS was histopathologically confirmed by the presence of intraneuronal Bunina bodies. Specimens of cervical (C3-C6 segments) and lumbar spinal cords (L4–L5 segments) from ALS cases and disease control cases were obtained. 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.

Fractionation of spinal cord and brain samples

Snap-frozen of spinal cords and various regions of brains were fragmented with TBS, ultra-centrifuged and then stored frozen as the “TBS-soluble fraction”. The resulting pellet was resuspended in TBS containing 1% SDS. The pellet was dissociated, centrifuged and stored frozen as the “TBS-insoluble/1% SDS-soluble fraction”. Alternatively, tissues were lysed in 1% Triton-X 100 and used for enzymatic treatments.

Immunoblots, Immunohistochemistry, and Immunoelectron microscopy

All experiments were performed according to standard protocols.

Statistical analysis

All data are presented as mean ± SD unless otherwise noted. The values were analyzed by unpaired Student's t-test.Differences in survival times were analyzed with Kaplan–Meier survival statistics (log-rank test; SPSS Software, IBM Japan, Tokyo, Japan).

Results

Characterization of 5D4-reactive keratan sulfate

In SOD1^{G93A} mouse spinal cord extracts, expression of the 5D4 KS epitope was increased compared with that in the non-transgenic controls (Figure 1A). To characterize the 5D4 epitope, lysate samples of SOD1^{G93A} spinal cords were pretreated with α2-3,6,8 neuraminidase (sialidase), α1-3,4 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. Pre-treated with a mixture of the neuraminidase,fucosidase, and subsequently treated with keratanase II,the 5D4 immunoreactivitywas reduced to an undetectable level (Figure 1B).Next, in order to determine whether the 5D4-reactive KS is linked through N-glycan or not, lysate of SOD1^{G93A}spinal cords was incubated with PNGase F and then subjected to Western blotting analysis. 5D4 immunoreactivity was comparable to that of the control sample. ß-Elimination reaction abolished the 5D4 immunoreactivity in the sample (data not shown).We found that the Mac2/galectin3 signals (90%), a proliferating microglia marker, were mostly colocalized with 5D4-KS signals (Figure 1C). Pre-embedding immunoelectoron microscopy showed that 5D4-KS signals were seen in cell surface membrane structures of microglia and their processes (Figure 1D).

Sulfotransferases required for 5D4-reactive keratan sulfate in SOD1^{G93A} mice

To understand possible mechanisms of up-regulation of the 5D4 epitope in the spinal cord of the ALS model mouse, mRNA expression levels of KS synthesis enzymes were measured by quantitative PCR. We found that GlcNAc6ST1 and ß1,3-N-acetylglucosaminyltransferase 7 (ß3GnT7) were significantly increased 3.5- to 4-fold in SOD1^{G93A} spinal cords comparable to those of the non-transgenic controls (data not shown). Immunoblotting with an anti-GlcNAc6ST1 or an anti-KSGal6ST antibody showed that GlcNAc6ST1 protein was increased 20-fold in SOD1^{G93A} mice while that of KSGal6ST was comparable to the level of the non-transgenic mice (Figure 2A).Disruption of sulfotransferase enzymes in SOD1^{G93A}/GlcNAc6ST1$^{-/-}$ and SOD1^{G93A}/KSGal6ST$^{-/-}$ mice did not show expression of the 5D4 epitope (Figure 2B).As we observed in the previous report (Hirano et al *PLoS ONE* 2013), a lifespan of SOD1^{G93A}/GlcNAc6ST1$^{-/-}$ mice was significantly shortened compared with SOD1^{G93A} mice,while SOD1^{G93A}/KSGal6ST$^{-/-}$ mice showed a lifespan similar to that of SOD1^{G93A} mice

(Figure 2C). We also found that 5D4-reactive KS is abundantly expressed in the brainstem but not in the frontal motor cortexof SOD1^{G93A} mice at disease end-stage (Figure 2D).

Elicited 5D4-reactive keratan sulfate in postmortem spinal cords of ALS patients

Postmortem cervical and lumber spinal cords of non-ALS disease-controls and sporadic ALS patients were immunoblottedwith 5D4.Immunoreactive smear bands with molecular weights of > 400 kDa and >250-150 kDa were detected in all cases of ALS tested. These bands were not detectable in all three disease control specimens (Figure 3).

Discussion

In this study, it has been found that the 5D4-reactive KS glycans expressed in the spinal cord of SOD1^{G93A} mice at the end-stage becomes susceptible to keratanase II upon 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-ß-galactosidase well supports this prediction (data not shown).The susceptibility to ß-elimination but not PNGase F treatment indicated that the 5D4-reactive KS is elongated from an *O*-GalNAc or *O*-Man linkage. Mac2/galectin-3 immunoreactivity is detected in activated/proliferating microglia associated with chronic or acute neuronal deficits, but absent in quiescent microglia (Lalancette-Hebert et al, *J Neurosci*2007). The 5D4-reactive KS is expressed in Mac2/galectin-3-positive microglia in the SOD1^{G93A} spinal cord and brainstem. The 5D4 epitope is expressed in a subpopulation of microglia that are CD86-positive (Hirano et al, *PLoS ONE* 2013). 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 (Linnartz et al, *Cell Tissue Res* 2012). Immunoelectron microscopy observations suggested that the 5D4-reactive KS might also be involved in microglial surveying and sensing of the local environment by contacting other cells.

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 (Chiu et al *Cell Rep* 2013). 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 indicates that both GlcNAc6ST1 and KSGal6ST are required for the synthesis of the microglial 5D4 KS epitope. 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. 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/bulbar lower motor neurons, but not in corticospinal upper motorneurons. In accordance with these results, 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.

Conclusion

The 5D4-reactive KS are expressed in central nervous tissues of SOD1^{G93A} mice and patients with ALS. The KS are modified with sialic acid and fucose. In the spinal cord of SOD1^{G93A} mice, GlcNAc6ST-1 is up-regulated while KSGal6ST is not.GlcNAc6ST1 and KSGal6ST are essential for biosynthesis of the 5D4-reactive KS in the central nervous tissues of SOD1^{G93A} mice and that expression of the microglial 5D4-KS may be related to degeneration of lower spinal/bulbar motor neurons in ALS pathogenesis.