DISCUSSION

PRNP polymorphisms are associated with variation in susceptibility to prion disease in humans [24], sheep [2, 4, 5, 13] and mice [34]. There have been many studies of relationships between *PRNP* polymorphisms and susceptibility to BSE in cattle [8, 12, 14, 22, 25, 28, 29, 31, 33]. However, few studies have found significant relationships between *PRNP* polymorphisms and occurrence of BSE [28]. The number of BSE-affected animals tested in the present study (6 cattle) was too small for reliable estimation of genetic relationships between bovine *PRNP* polymorphisms and occurrence of BSE, and we found no evidence of such relationships.

In the present study, we assayed for *PRNP* polymorphisms in 863 healthy Holstein cattle and 186 healthy JB cattle. The only polymorphism caused amino acid substitutions in the ORF was the octapeptide repeat. We observed only 2 SNPs, at G234A and C576T; these SNPs have frequently been observed in other studies [8, 10, 13, 17, 28, 31]. Only 2 of the 863 Holstein cattle were heterozygous for the C576T polymorphism, whereas 91 of the 186 JB cattle were heterozygous at C576T (Table 2). Similar differences in allele frequencies between the 2 cattle breeds were found at the 23- and 12-bp indels and the G234A SNP. These differences may be due to inherent properties of the cattle breeds or differences in the breeding systems used for Holstein and JB cattle. The allele frequencies of the bovine *PRNP* gene observed in the present study for JB cattle suggest a breeding system in which a limited number of bulls is used. Takasuga *et al.* [31] found 13 SNPs (including 2 amino acid substitutions) in indigenous Indonesian cattle, which carry more mutations than Holstein and JB cattle. It is generally thought that artificial insemination, which is widely used to breed dairy and beef cattle, decreases genetic variation and produces uniform genetic properties at the DNA level. Holstein and JB cattle raised in Japan appear to have fewer mutation sites in the *PRNP* gene than indigenous breeds such as indigenous Indonesian cattle [31].

Several cattle breeds have been shown to have octarepeat polymorphisms in the ORF of the *PRNP* gene [5, 22, 25, 33], and 3 alleles of the octapeptide repeat (5, 6 and 7 copies) have been reported. In the present study, none of the cattle had 7 copies of the octapeptide repeat, and the frequency of the 5-copy allele was very low (Tables 1 and 2). The predominant genotype of the octapeptide repeat in the present Holstein and JB cattle was homozygosity of the 6-copy allele. Variability of the octapeptide repeat has not been found to correlate with incidence of BSE in cattle [5, 14, 23], and no BSE-affected cattle have been found to be homozygous for the 5-copy allele. We found no significant differences in the genotype distribution of the SNPs or octarepeat polymorphisms between healthy and BSE-affected Holstein cattle (Table 2).

In the present study, we investigated DNA polymorphisms in 2 regions upstream from the ORF: a 23-bp indel in the upper region of exon 1, and a 12-bp indel in intron 1 (Fig. 1). The available evidence suggests that polymorphisms in these regions affect transcription of the *PRNP* gene [15, 18, 21]. In a previous study, the 23-bp insertion was found to occur more frequently in healthy cattle than BSE-affected cattle [28]. Among Holstein cattle in Japan, the 23-bp insertion has been found to have a lower allele frequency than the 23-bp deletion. We speculated that polymorphism of the 12-bp indel might affect expression levels of the *PRNP* gene, because the indel is in the promoter region of intron 1 and contains a putative Sp1-binding consensus sequence [9, 15, 18, 19]. It has been reported that a GC-rich region and Sp1-binding sequence upstream of exon 1 are both important factors in PRNP transcription [1, 15, 18], but the effects of this Sp1 sequence in intron 1 are unclear. Further research is needed to clarify the effects of the12-bp indel on expression of the *PRNP* gene.

In the present study, we identified a *PRNP* gene mutant with a 288-bp deletion in the ORF (Figs. 1 and 2D), in a specimen from our DNA stock samples. A prion protein with such an internal deletion may confer resistance to prion disease infection or act as a dominant-negative mutant that inhibits prion propagation in the cell [17, 36]. Accurate determination of the distribution of this deletion could help clarify whether it has a preventive effect against prion disease. However, we did not detect this 288-bp deletion in the *PRNP* gene of any of the present 1049 cattle. Our stock DNA specimen with the 288-bp deletion in the *PRNP* gene was obtained from a calf (C928) with the calf form of sporadic bovine leucosis (SBL) [16]. SBL appears to be caused by a somatic mutation in immature pre-B cells, and it is unclear whether the malignant process of SBL is related to an internal deletion in the *PRNP* gene. If the internal deletion in the *PRNP* gene found in specimen C928 is caused by a somatic mutation that is related to the malignant transformation of SBL, it is unlikely that such a deletion would be found in healthy cattle. We examined the bovine *PRNP* gene from the other 7 specimens of SBL calves, but none of those specimens had the same deletion (data not shown).

In conclusion, we found an extremely small number of *PRNP* polymorphic sites in the 2 cattle breeds examined in the present study, and we found no association between these polymorphisms and BSE. Because the number of BSE-affected animals tested was small, further genetic investigations using many samples from BSE cattle can be useful for assessment of the risk of BSE in Japan.

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FULL PAPER

Alymphoplasia mice are resistant to prion infection via oral route.

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Abstract

The major cause of infection in animal prion diseases is thought to be consumption of prion-contaminated stuff. There is evidence that the enteric nerve system (ENS) and gut-associated lymphoid tissues (GATL) are involved in the establishment of prion infection through alimentary tract. To elucidate the initial entry port for prion, we inoculated prion to alymphoplasia (*aly*) mice showing a deficiency in systemic lymph nodes and Peyer's patches. The *aly/aly* mice were susceptible to prion infection by intra-cranial inoculation and there were no differences in incubation periods between *aly/aly* mice and wild-type C57BL/6J mice. Incubation periods in *aly/aly* mice were about 20 days longer than those in C57BL/6J mice with the intra-peritoneal inoculation. The *aly/aly* mice were completely resistant to prion infection by per os administration, while C57BL/6J mice were sensitive as they entered the terminal stage of disease around 300 days post inoculation. PrP^{Sc} were detected in the intestine and spleen of C57BL/6J mice inoculated with prion intraperitoneally or orally ; however PrP^{Sc} was not detected in the spleen and intestine of *aly/aly* mice. Prion infectivity was detected in the intestines and spleens of prion-inoculated C57BL/6J mice, even after the early stages of exposure, while no infectivity was detected in these tissues of prion-inoculated *aly/aly* mice. No apparent differences were observed in the organization of the enteric nerve system between wild-type and *aly/aly* mice. These results indicate that GALT rather than ENS acts as the primary entry port for prion after oral exposure.

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Key words : prion, scrapie, alymphoplasia, GALT

Introduction

Transmissible spongiform encephalopathies (TSEs or prion diseases) are a group of fatal neurodegenerative diseases that include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting diseases (CWD) in deer and elk, and Creutzfeldt-Jakob disease in humans. Prion diseases have a long asymptomatic incubation period followed by a relatively short clinical phase, and they are characterized by the accumulation of disease-specific , protease-resistant isoforms of prion protein (PrP) , designated PrP-res or PrP^{Sc}, in the central nervous system (CNS). PrP^{Sc} is post-translationally generated from the normal , protease-sensitive isoform of PrP, designated PrP-sen or PrP^{C}, which is expressed in many tissues and is particularly strongly expressed in the CNS. Although PrP^{Sc} is derived from host gene-encoded normal host protein, PrP^{C}, a line of evidences suggests that PrP^{Sc} is a major component of the TSE agent.

Although the CNS is the only site of histopathologically discernible damage, the port of entry for exogenous prion in animal prion diseases such as scrapie, BSE, and CWD is thought to be an alimentary tract. The route of neuroinvasion of prion has been well documented using prion-infected rodent models ; there are at least two pathways for neuroinvasion, one is retrograde along the parasympathetic fibers of the vagus nerve to the medulla oblongata, and the other is along the sympathetic fibers of the splanchnic nerve to the thoracic/lumbar spinal cord.[1-4] The existence of the two pathways to CNS is also confirmed by the extensive immunohistochemical analysis of naturally occurring sheep scrapie.[5] In the early stages of oral exposure to prion, PrP^{Sc} can be detected in the nerve cells of enteric nerve system (ENS) and follicular dendritic cells (FDC) of tonsil and other submucosal lymphoid follicules in the alimentary tract.[6-8] These data indicate that ENS and Gut-associated lymphoid tissues (GALT) are the initial entry port for prion infection. However, it is unclear which of these is primarily important for the establishment of prion infection in the alimentary tract and subsequent neuroinvasion..

The alymphoplasia (*aly*) mutation in mice is autosomal recessive and is characterized by a deficiency in systemic lymph nodes and Peyer's patches.[9] Recently, the *aly* allele was found to carry a point mutation causing in amino acid substitution in the carboxy-terminal of NF-κB inducing kinase (NIK).[10] Due to the lack of Peyer's patches, *aly* mice provide a suitable model for analyzing the involvement of GALT in the initial entry of prion in the alimentary tract. To elucidate the involvment of ENS and GALT in the establishment of prion infection, we analyzed the prion susceptibility of *aly* mice with various routes of infection. The results indicate GALT is a key tissue for the establishment of prion infection through the oral route.

Materials and Methods

Mice

ALY/NscJcl-*aly* (*aly*/*aly*) mice and their wild-type, C57BL/6J mice, were purchased from CLEA Japan Inc. ICR mice for bioassay were also purchased from CLEA Japan Inc.

Experimental inoculation

Mouse-adapted scrapie Obihiro strain was propagated in ICR mice. After entering the terminal stage of the disease, mice were sacrificed under anesthesia and brains were

collected. The brains were used as a source of brain homogenate for experimental inoculation. For intra-cranial (i.c.) inoculation, 20 μl of 1 % brain homogenate was injected into the left hemisphere. Mice assigned to the intraperitoneal (i.p.) inoculation group received 100 μl of 0.1% brain homogenate. Oral administration (p.o.) was carried out as described by Maignien et al.[11] Animals were placed in individual cages equipped with a liquid delivery system consisting of a 1.5-ml Eppendorf tube with a 3-mm hole at the bottom. Tubes were filled with 100 μl of a mixture of Endolipid (20% soya oil, 1.2% egg lecithin, 2.5% glycerol in water) and 20% brain homogenate (Endolipid : brain homogenate =1:1). Consumption of the infectious preparations was individually monitored.

Bioassay

During the course of experimental infection, 2 mice were sacrificed at each time point, and their brains, spleens and intestines were collected. Fifty milligrams of each tissue from the two mice was pooled and homogenized in PBS (10% w/w) a Multi-beads shocker (Yasuikiki, Japan) at 2,000 rpm for 1 min, followed by sonication for 30 sec. ICR mice were inoculated by i.c. route with 20 μl of the tissue homogenates and were observed until they exhibited the clinical symptoms of the terminal stage.

Detection of PrP^{Sc}

Samples were prepared as described previously,[12] with minor modifications described below. Minced tissues were homogenized in about eight volumes of buffer consisting of 2 % (v/v) Zwittergent 3-12, 0.5% sodium deoxycholate, 100 mM NaCl, and 50 mM Tris-HCl (pH7.5). Homogenates were digested with collagenase (0.5mg/100mg tissue) and DNase I (40μg/100mg tissue) with constant rotation at 37℃ for 6 to 12 hr until lumps of tissue were dispersed. Proteinase K (50 μg/100 mg tissue) was then added, and the homogenates were further incubated for 1 hr. Pefabloc was added at a final concentration of 2 mM to stop proteinase K digestion. Samples were then centrifuged at 68,000 g for 40 min at 20℃. The pellets were then dissolved in eight volumes (relative to the starting tissue sample) of 6.25% Sarkosyl in Tris-HCl, pH 8.0. After incubation at 37℃ for 30 min, the samples were centrifuged at 12,000g for 5 min at 20℃. Supernatants were a brought to 12% (w/v) NaCl, and were centrifuged at 100,000g for 40 min at 4 ℃. The resulting pellets were dissolved in sodium dodecyl sulfate-polyacrylaminde gel electrophoresis (SDS-PAGE) sample buffer (5% SDS, 4M Urea, 5% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, 62.5 mM Tris-HCl, pH 6.8). Immunoblot was performed as described elsewhere with some modifications.[13] SDS-PAGE was carried out using 14% Bis-Tris gels (Invitrogen) according to the supplier's instruction. Western transfer to Immobilon-P transfer membranes (Millipore) was carried out using the Trans-blot mini cell (Bio-Rad) at 60 V for 2 hr. After transfer, membranes were blocked for 1 hr at room temperature with 5% skim milk in phosphate-buffered saline (PBS) containing0.1% Tween 20 (PBST) and were then incubated for 1 hr with primary antibodies diluted in the 1% skim milk in PBST. Blots were then washed with PBST and incubated with secondary antibody for 1 hr. Blots were visualized using an ECL Western blot detection kit (Amersham).

Immunochistochemical (IHC) detection of PrP^{Sc} was carried out as described elsewhere.[14] B103 polyclonal antibodies against bovine PrP synthetic peptide were used for detection.[15]

Staining of ENS

Tissues were fixed with 10% formalin, 0.2% picric acid in PBS, and wholemount specimens or cryosections (20 μm) were stained with antibodies against Protein Gene Product 9.5. Antibody reactivity of the antibody was visualized using the avidin-biotin complex method as described elsewhere.[16]

Results

Susceptibility of aly/aly mice to prion via various route of inoculation

In order to examine the susceptibility of *aly/aly* mice to prion infection, we inoculated *aly/aly* and C57BL/6J mice with brain homogenates of scrapie-infected mice via i.c., i.p. or p.o. route. Table 1 shows the incubation periods to reach the terminal stage of disease. No significant differences were observed in the incubation periods between *aly/aly* mice (159 days) and C57BL/6J mice (165 days) when they were inoculated with prion via i.c. route, and no apparent differences in clinical manifestation were seen between aly/aly and C57BL/6J mice. Furthermore, no differences were observed in the accumulation of PrP^{Sc} in the mouse brains (Fig. 1) or in neurohistopathological findings (data not shown), indicating that the *aly* phenotype, which is caused by a point mutation in NIK, dose not influence the neuropathogenesis of prion diseases nor prion replication in the CNS.

In i.p. inoculation, the incubation period in *aly/aly* mice was prolonged by 28 days, as compared with C57BL/6J mice, but all mice developed the typical clinical symptoms of scrapie. Although PrP^{Sc} levels in the brain were the same, there was a striking difference in the accumulation of PrP^{Sc} in spleen; PrP^{Sc} was not detected in the spleens of scrapie-affected *aly/aly* mice (Fig. 1). The severe combined immunodeficiency (SCID) mouse spleen did not support prion replication due to the lack of mature FDC, and SCID mice were found to be resistant to prion when low doses of prion were administered i.p. In contrast, SCID mice developed clinical symptoms with-

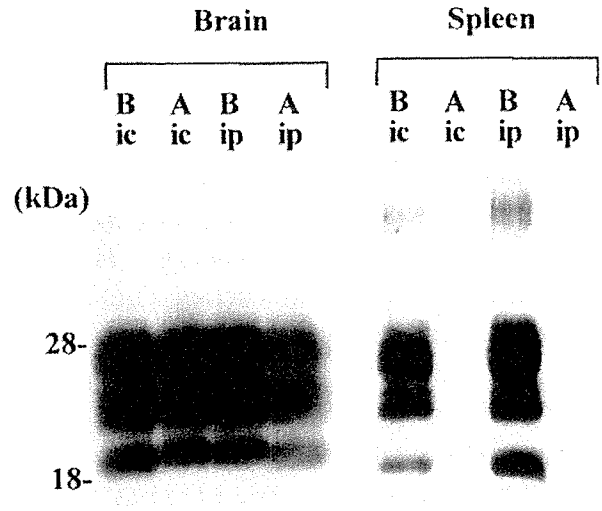

Fig. 1. Detection of PrP^{Sc} in brain and spleen. Mice exhibiting symptoms of the terminal stage of disease were sacrificed and examined for PrP^{Sc}. B, C57BL/6J mice; A, *aly/aly* mice. i.c., inoculated by intra-cranial route; i.p., inoculated by intra-peritoneal route. Molecular mass markers are in kilo Daltons.

Table 1. Susceptibility of aly/aly mice to prion exposure via various routes.

Route	Concentration, amount of homogenate	Infectious dose[1] (LD_{50})	Period to terminal stage (days, mean ± SD)	
			C57BL/6J (Attack rate)	*aly/aly* (Attack rate)
i.c.	10%, 20 μl	10^6	165±5 (4/4)	159±8 (4/4)
i.p.	0.1%, 200 μl	10^5	251±9 (6/6)	279±6 (4/4)
p.o.	10%, 100 μl	5×10^6	307±7 (7/7)	>700 (0/5)

[1]Infectious doses were expressed as 50% lethal dose (LD_{50}).

out any PrP accumulation in the spleen when moderate doses of prion were administered i.p., although the incubation periods were longer than in wild-type mice.[17,18] This difference can be explained by direct spreading to the CNS from peripheral nerves. The prolonged incubation periods in *aly/aly* mice inoculated with prion via i.p route, without accumulation of PrP^{Sc} in spleen, could thus be explained by the same mechanism.

Obvious differences in susceptibility were observed when mice were challenged by p.o. route ; all of wild-type mice entered the terminal stage within 307±7 days post infection (dpi) ; however, none of the *aly/aly* mice showed clinical symptoms and remained healthy throughout the experimental period (700days). IHC analysis showed that PrP^{Sc} was present in the GATL of C57BL/6J mice at the terminal stage of the disease but was not present in that of *aly/aly* mice at the end of the experiment (Fig. 2). These results indicate that *aly/aly* mice are susceptible to prion replication in the CNS, but that neuroinvasion did not take place via oral consumption.

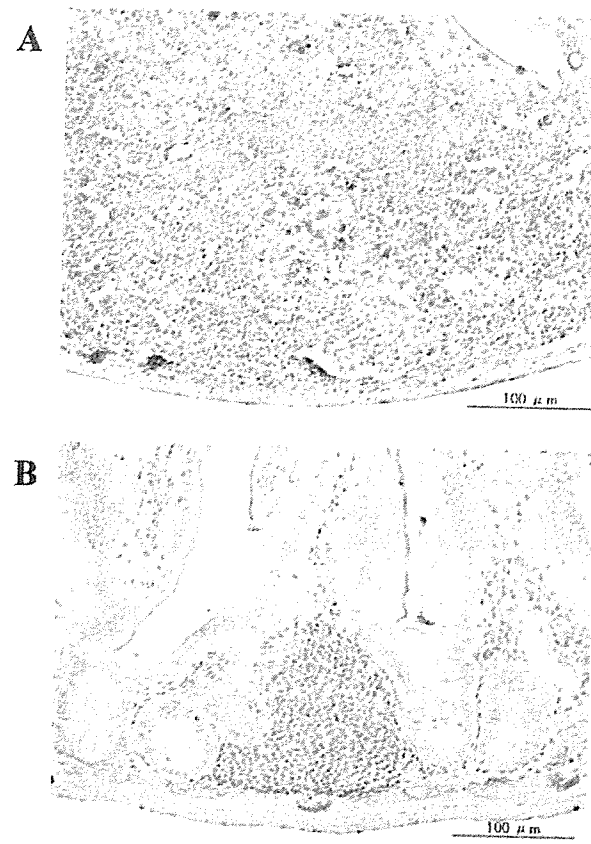

Fig. 2. Immunohistochemical detection of PrP^{Sc} in the ileum.
The ilea of mice inoculated p.o. were examined for PrP^{Sc}. (A) C5BL/6J mice at 309 dpi. (B) *aly/aly* mice at 700 dpi.

Prion infectivity in alimentary tract and spleen

In an effort to determine whether uptake and replication of prion occur in the alimentary tract of *aly/aly* mice, prion infectivity in the intestine was analyzed by bioassay. In the p.o. group, two mice from each mouse strain were sacrificed at 19, 43, and 83 dpi and tissue homogenates were inoculated i.c. into ICR mice for bioassay. Infectivity was detected in the intestines of C57BL/6J mice at each time point, however, no infectivity was detected in the intestines of *aly/aly* mice (Table 2), suggesting that prion did not replicate in the intestine of *aly/aly* mice. Infectivity was not detected in the spleens of *aly/aly* mice inoculated p.o., while considerable amounts of infectivity were detected in the spleens of the corresponding C57BL/6J mice at 80 dpi. Furthermore, traces of infectivity were detected in the spleens of *aly/aly* mice, even after i.p. inoculation ; one of five mice manifested the terminal stage of the disease at 381 dpi (Table 2) and the brain of this mouse was positive for PrP^{Sc} (data not shown), indicating that the lymphoid tissues of *aly/aly* mice did not support prion propagation.

Organization of ENS in aly/aly mice

Data described above suggested that GALT is essential as an initial entry port for prion infection, however, no information was available regarding the influence of NIK mu-

Table 2. Prion infectivity in spleen and ileum.

Group	dpi[1]	C57BL/6J		*aly/aly*	
		Spleen	Ileum	Spleen	Ileum
p.o.	19	NT[2]	246±36 (6/6)[3] 6.3×10^3	NT	>300 (0/5)
	43	NT	220±18 (4/4) 3.7×10^4	NT	>425 (0/5)
	83	176±4 (5/5) 8.9×10^6	307±21 (5/5)[4] 6.2×10^2	>450 (0/5)	>450 (0/5)[4]
i.p.	80	188±31 (4/4) 2.0×10^6	NT	>450 (0/4) 381 (1/5)	NT

[1]Spleens and/or ilea were collected from C57BL/6J or *aly/aly* mice at indicated days post infection.
[2]Not tested.
[3]Upper column shows incubation periods (days, mean ± SD) and attack rates (in parenthesis) of mice used for bioassay, while lower column shows estimated infectivity (LD_{50}/g tissue) from the incubation periods.
[4]Ileum homogenates of the group were treated at 60℃ for 30 min before inoculation to mice for bioassay.

tation on the organization of ENS. Therefore, we analyzed the organization of ENS by immunohistochemistry with protein gene product 9.5 as an ENS marker (Fig. 3). No obvious deficiency in organization of nerve fibers, or submucosal and intramuscular plexus was observed in *aly/aly* mice, supporting the idea that GALT is a primary target for prion entry via oral consumption.

Discussion

Although the pathway of invasion into CNS is well characterized,[1,3-5] it remains unclear how prion enter the host, particularly under natural circumstance. The major route of infection in naturally occurring prion diseases in animals is believed to be oral consumption of the infectious agent. Accumulating evidence suggests that the ENS and GALT are the primary target sites for prion entry and replication in peripheral tissues.[1,3,4] We therefore attempted to address the question of whether the ENS or GALT is important in the establishment of prion infection via the oral route. Our results demonstrated that GALT is essential for initial uptake of prion from the gut lumen to the alimentary tract. No differences were observed

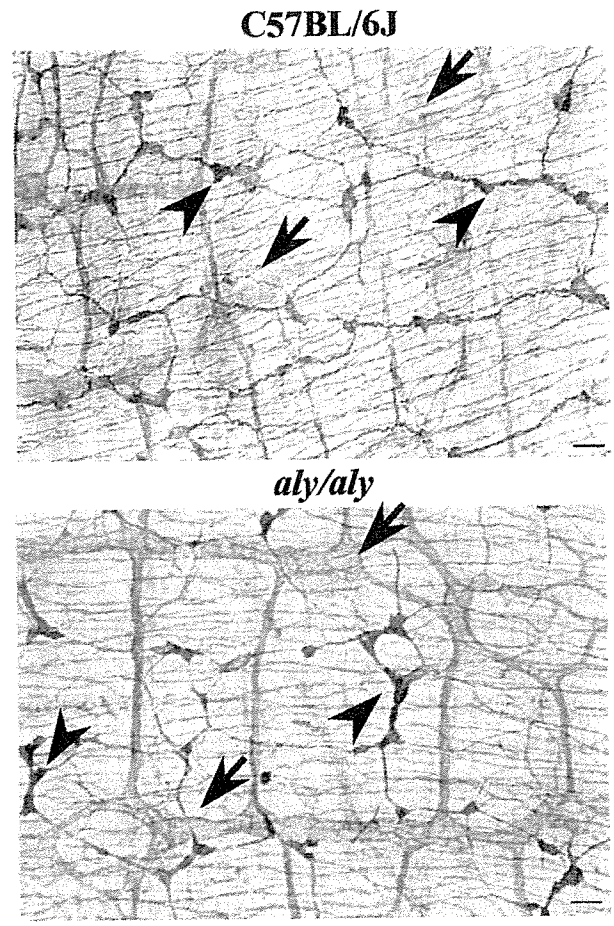

Fig. 3. Organization of enteric nerve system. Extended intestine specimens were stained with anti-protein gene product 9.5 antibodies. Arrowheads indicate submucosal plexus, whereas arrows indicate intra-muscular plexus. Bar : 50 μm

in the organization of ENS between *aly/aly* and C57BL/6J mice, suggesting that prion adsorption via the epithelial cells of digestive tract and subsequent entrance into peripheral nerve fibers or blood stream is unlikely.

The follicle-associated epithelium (FAE) that covers the dome of Peyer's patches or submucosal lymphoid follicle contains villus and M cells. M cells act as major ports of entry for enteric pathogens via trans epithelial transport[19], and Heppner *et al.* reported that prion could be transported from the apical to the basolateral compartment in an *in vitro* model comprising epithelial cells morphologically and functionally resembling M cells.[20] It was reported that functional B cells are required for the development of the FAE, Payer's patches and M cells.[21] B cells of *aly/aly* mice are functionally impaired so that *aly/aly* mice may have reduced M cell numbers and/or functionally impaired M cells in FAE. Mice deficient in B lymphocytes (μMT mice), both B and T lymphocytes (RAG-$1^{-/-}$ mice), or in tumor necrosis factor and lymphptoxin-α, in which the number of Peyer's patches is reduced, were resistant to oral prion challenge.[22] In contrast, β7 integrin-deficient mice, in which B cells in Peyer's patches are severely reduced, which also possess normal numbers of Peyer's patches, were sensitive to oral prion infection.[22] The difference in prion susceptibility among those B cell-deficient mice following oral inoculation may be explained by the numbers of Peyer's patches and M cells.[22] The *aly/aly* mice were highly resistant to oral prion infection, similar to RAG-$1^{-/-}$ and μMT mice, and thus it is of interest to determine whether the presence of M cells and M cell function in *aly/aly* mice are involved in the initial entry of prion into the gastro-intestinal tract.

One of the interesting questions is why ruminants appear sensitive to prion via oral exposure. Anatomical and histological characteristics might explain the susceptibility of ruminants to prion. The ileal Peyer's patches are large organs in young lambs, extending for up to 2.5 cm, are estimated to contain over 100,000 follicles,[23] and develop well in the distal ileum. FEA of cattle was reported to contain higher M cell or M cell-like populations than that of rodents.[23,24] These features suggest that the intestines of ruminant possess more ports of entry for prion than those of rodents. The scrapie susceptibility of sheep is thought to decline with growth.[25] The involution of the ileal Peyer's patch at puberty and the accompanying drastic reduction in the number of follicles and FAE may contribute to the reduced susceptibility to prion that is observed in older animals.

PrP^{Sc} and prion infectivity can be readily detected in the lymphoreticular tissues of scrapie-infected sheep, in contrast, PrP^{Sc} and prion infectivity were scarcely detected in the lymphoreticular tissues of BSE-affected cattle.[26] However, PrP^{Sc} has been detected in the ENS of BSE-affected cattle (Iwata et al., submitted for publication). Thus efficient replication of BSE agent in peripheral lymphoreticular tissues may not be essential for the neuroinvasion of BSE agent after oral exposure. The *aly/aly* mice possess normal ENS organization but are resistant to prion infection via the p.o. route, suggesting that the ENS does not act as a port of entry for prion after oral exposure.

Acknowledgements

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Inhibition of PrP^{Sc} formation by synthetic *O*-sulfated glycopyranosides and their polymers

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Abstract

Sulfated glycosaminoglycans (GAGs) and sulfated glycans inhibit formation of the abnormal isoform of prion protein (PrP^{Sc}) in prion-infected cells and prolong the incubation time of scrapie-infected animals. Sulfation of GAGs is not tightly regulated and possible sites of sulfation are randomly modified, which complicates elucidation of the fundamental structures of GAGs that mediate the inhibition of PrP^{Sc} formation. To address the structure–activity relationship of GAGs in the inhibition of PrP^{Sc} formation, we screened the ability of various regioselectively *O*-sulfated glycopyranosides to inhibit PrP^{Sc} formation in prion-infected cells. Among the glycopyranosides and their polymers examined, monomeric 4-sulfo-*N*-acetyl-glucosamine (4SGN), and two glycopolymers, poly-4SGN and poly-6-sulfo-*N*-acetyl-glucosamine (poly-6SGN), inhibited PrP^{Sc} formation with 50% effective doses below 20 μg/ml, and their inhibitory effect became more evident with consecutive treatments. Structural comparisons suggested that a combination of an *N*-acetyl group at *C*-2 and an *O*-sulfate group at either *O*-4 or *O*-6 on glucopyranoside might be involved in the inhibition of PrP^{Sc} formation. Furthermore, polymeric but not monomeric 6SGN inhibited PrP^{Sc} formation, suggesting the importance of a polyvalent configuration in its effect. These results indicate that the synthetic sulfated glycosides are useful not only for the analysis of structure–activity relationship of GAGs but also for the development of therapeutics for prion diseases.

Keywords: Prion; Transmissible spongiform encephalopathy; Glycosaminoglycan; Sulfated glycosides

Transmissible spongiform encephalopathies (TSEs), so-called prion diseases, are neurodegenerative diseases with long incubation periods and invariably fatal outcomes. Prion diseases include Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträusser-Schinker syndrome (GSS) in human beings, scrapie in sheep and goats, and bovine spongiform encephalopathy. One of the characteristics of TSEs is an accumulation of a protease-resistant, abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system. PrP^{Sc} is posttranslationally generated from the host-encoded, protease-sensitive prion protein (PrP^{C}) [1]. A central event in the pathogenesis of TSEs is the conversion of PrP^{C} to PrP^{Sc} [1]; therefore, it is expected that inhibition of PrP^{Sc} formation will be an effective way of treating prion diseases.

It is well known that sulfated glycosaminoglycans (GAGs) and sulfated glycans such as dextran sulfate 500 (DS500) and pentosan polysulfate (PPS) prevent prion infection via the peripheral route when administered prior

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to, simultaneously, or just after the inoculation with prion [2–5]. They also have been shown to inhibit PrP^{Sc} formation in scrapie-infected murine neuroblastoma cells [6,7]. Based on these findings, sulfated GAGs and their analogues have been considered as candidates for the development of therapeutics for treating prion diseases. Recently, Doh-ura et al. reported that intraventricular infusion of PPS prolonged the survival time of prion-infected mice, antagonized PrP^{Sc} accumulation and also reduced neuronal degeneration even when the infusion was given at the late stage of infection [8]. Clinical trials of intraventricular infusion of PPS to CJD and GSS patients have been started in some countries including the United Kingdom.

GAGs consist of a number of disaccharide repeating units, which are composed of uronic acid (glucuronic or iduronic acid) and an amino sugar (galactosamine or glucosamine). The uronic acid and the amino sugar have one to two and two to three possible sites of sulfation, respectively, although these sites are not always sulfated. Consequently, the various combinations of sulfations yield many different possible disaccharide units [9,10]. Although sulfated GAGs may be useful for treating prion diseases, core structures necessary for inhibition of PrP^{Sc} formation are still unclear. The identification of the core structures should help in the development of compounds with enhanced therapeutic potential.

To examine the structure–activity relationship (SAR) of GAGs in the inhibition of PrP^{Sc} formation, we screened various regioselectively *O*-sulfated glycopyranosides as mimics of GAGs and their components [11–13]. Here we show that some synthetic sulfated glycopyranosides and their polymers inhibit PrP^{Sc} formation in prion-infected cells. The results presented here suggest that the locations of the *O*-sulfate and *N*-acetyl groups on glucopyranosides are important for the inhibition of PrP^{Sc} formation.

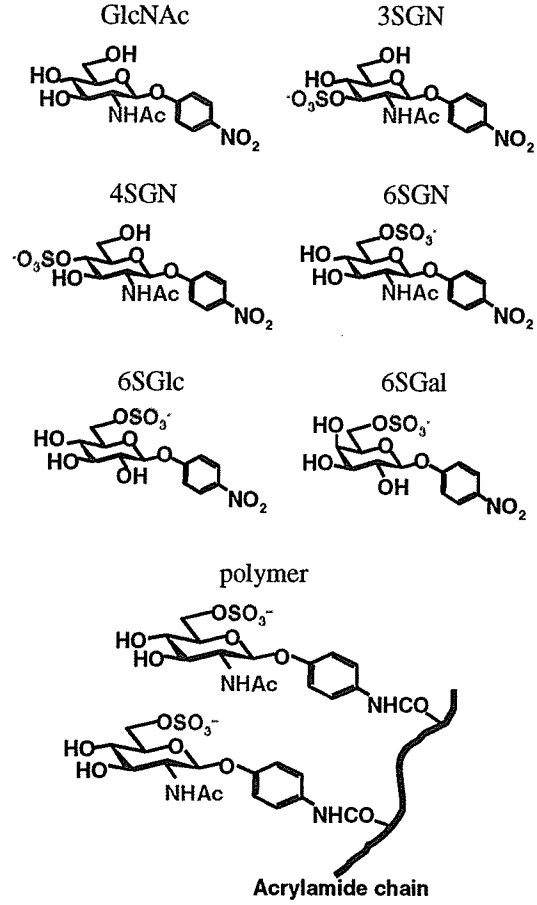

Fig. 1. Structures of sulfated glycopyranosides and their polymers. Structures of mono-glycopyranosides used in this study. 3SGN, 4SGN, 6SGN, 6SGal, 6SGlc, and GlcNAc were also used in polymeric form, in which the glycopyranosides were coupled to an acrylamide chain through their *p*NP residues [14].

Materials and methods

Glycopyranosides and their polymers. The structures of glycopyranosides used in this study are shown in Fig. 1. Six monomeric (mono-) *p*-nitrophenyl (*p*NP) glycosides, *p*NP *N*-acetyl-glucosaminide (GlcNAc), *p*NP 3-sulfo-GlcNAc (3SGN), *p*NP 4-sulfo-GlcNAc (4SGN), *p*NP 6-sulfo-GlcNAc (6SGN), *p*NP 6-sulfo-glucopyranoside (6SGlc), and *p*NP 6-sulfo-galactopyranoside (6SGal) were used. In addition, we used polymers of the glycopyranosides, in which the mono-glycopyranosides were linked to acrylamide chains to mimic the oligosaccharide entity of GAGs [14]. Molar ratios of acrylamide to each glycopyranoside were approximately 9:1, indicating that each polymer has ca. 10% of glycopyranoside as residues. Average molecular weights of these polymers were estimated to be approximately $1.2–3.3 \times 10^5$. The compounds were dissolved in distilled water or dimethyl sulfoxide and filtered through a 0.45-μm Milex filter (Millipore). Heparan sulfate (HS) and heparin were purchased from Sigma. DS500 was purchased from Polysciences. Inc. PPS (Cartrophen Vet, Biopharm Australia Pty, Ltd.) was generously provided by Dr. Katsumi Doh-ura, Tohoku University.

Cell culture. Neuro2a mouse neuroblastoma cells (ATCC CCL-131) were cultured in Dulbecco's modified Eagle's medium (ICN Biomedicals) supplemented with 10% fetal bovine serum (FBS) and non-essential amino acids. Mouse neuroblastoma cells persistently infected with prion, which were originally established by Race et al. [15], were cloned by limiting dilution, and a resulting subclone (I3/I5-9) that possessed a high level of PrP^{Sc} [16], was used in this study. The I3/I5-9 cells were maintained in Opti-MEM (Invitrogen) containing 10% FBS, and cells passaged fewer than 20 times were used for the experiments.

Treatment of cells and sample preparation, SDS–PAGE, and immunoblotting. The I3/I5-9 cells or Neuro2a cells nearly confluent in a 25 cm^2 flask were seeded in a 35 mm tissue culture dish with 1:10 dilution. On the second day, the medium was replaced with 3 ml of Opti-MEM containing 10% FBS (for I3/I5-9 cells) or 3 ml DMEM containing 10% FBS (for Neuro2a cells) and test compounds were added to the medium. The cells were cultured for 2 days in the presence of test compounds and examined for the presence of PrP^{C} or PrP^{Sc} by immunoblotting as described previously [16]. For quantitative analysis, we used one of the following: the Western-Star™ Protein Detection Kit (Tropix) for chemiluminescent detection and quantitation of immunoreactive bands using an LAS-1000 lumino image analyzer (Fujifilm) as described previously [16]; or an ECL Western Blotting detection kit (Amersham Biosciences) and quantitation with an LAS-3000 lumino image analyzer (Fujifilm).

Indirect immunofluorescence assay (IFA). Cells seeded on 8-well chamber slides (Nunc) were treated with test compounds for 2 days and then fixed with methanol for 20 min at −20 °C. After blocking for 30 min with PBS containing 5% FBS (FBS–PBS), the cells were incubated for 1 h at room temperature with mAb 31C6 [17] diluted in 1% FBS–PBS. After washing with PBS, the cells were incubated for 1 h with 1:1,000 diluted Alexa 488-labeled Fab fragment of goat anti-mouse IgG. Finally, the slide was mounted with PBS containing 50% glycerol, and 1% *n*-propyl gallate

and examined with an Olympus IX71 fluorescence microscope equipped with a cooled CCD unit (*Cool*SNAP™ HQ, Roper).

Cell growth assay. Cells were seeded in 96-well plates at 1×10^4 cells/well in 200 μl of medium. On day 2, test compounds were added and the cells were incubated for 48 h. All experiments were carried out in quadruplicate. Next, 20 μl of a mixture of 1 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-1) and 0.2 mM 5-methylphenazinium methyl sulfate was added to each well and the plates were incubated for 3 h at 37 °C. Finally, the optical density at 450 nm was measured with a microplate reader. The relative growth ratio in the presence of test compounds was calculated by comparing the growth of cells with and without the test compounds.

Results

Effect of O-sulfated glycopyranosides on PrP^{Sc} formation in cells

To investigate the SAR of GAGs in the inhibition of PrP^{Sc} formation, we used regioselective *O*-sulfo glycopyranosides synthesized by a combination of chemical and enzymatic reactions (Fig. 1). Because GAGs are oligosaccharides consisting of uronic acids and amino sugars, we also used polymers of glycopyranosides. DS500, heparin, and PPS, which inhibit PrP^{Sc} formation in cultured cells [6], were used as positive controls.

Fig. 2 shows representative results of immunoblot analysis for the inhibition of PrP^{Sc} formation. A 2-day treatment with mono-4SGN, poly-4SGN, or poly-6SGN dose-dependently reduced PrP^{Sc} formation. In agreement with previous reports, DS500, heparin, and PPS reduced PrP^{Sc} formation, but HS did not [6,7]. Table 1 summarizes the effect of glycopyranosides on PrP^{Sc} formation from at least three independent experiments. Three glycopyranosides, mono-4SGN, poly-4SGN, and poly-6SGN, reduced PrP^{Sc} with low 50% effective dose (ED_{50}); ED_{50} of them were 10, 4, and 9 μg/ml, respectively. However, they were less potent than PPS ($ED_{50} = 0.3$ μg/ml) and DS500 ($ED_{50} = 0.5$ μg/ml), which are known to be the most effective GAG analogues for inhibiting PrP^{Sc} formation (Fig. 2 and Table 1). Other three glycopyranosides, mono-6SGal, poly-3SGN, and poly-6SGal, showed weak inhibitory activities ($ED_{50} > 20$ μg/ml; Table 1). We mainly focused on mono-4SGN, poly-4SGN, and poly-6SGN in the subse-

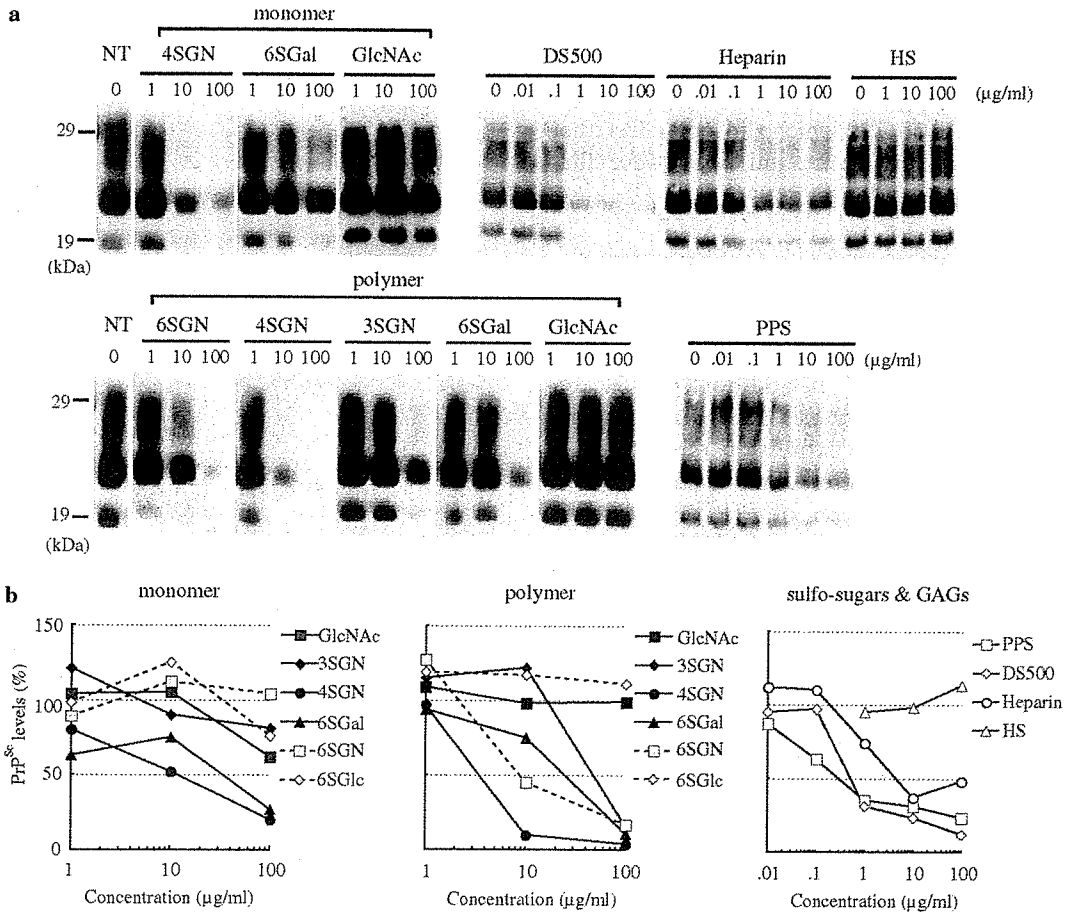

Fig. 2. Inhibition of PrP^{Sc} formation in prion-infected cells by sulfated glycopyranosides. (a) Representative results of PrP^{Sc} detection. I3/I5-9 prion-infected cells were treated for 2 days with various monomeric glycopyranosides and their polymers or GAGs (PPS, DS500, heparin, and HS) at the indicated concentrations. GlcNAc was included as a representative non-inhibitory glycoside. Western blots for the samples containing PPS, DS500, heparin, and HS were visualized with a LAS-3000 lumino image analyzer, whereas the samples containing the other compounds were detected with X-ray film. Molecular mass markers are indicated on the left. NT, untreated cells. (b) Quantitative analysis. Quantitative analyses were carried out using an LAS-1000 or an LAS-3000 lumino image analyzer. Results represent the average of at least three independent experiments.

Table 1
Effect of glycopyranosides and their polymers on PrP^{Sc} formation

Compound	ED_{50}[a] (μg/ml)
GlcNAc	>50
3SGN	>50
4SGN	10
6SGal	31
6SGN	>50
6SGlc	>50
Poly-GlcNAc	>50
Poly-3SGN	50
Poly-4SGN	4
Poly-6SGal	21
Poly-6SGN	9
Poly-6SGlc	>50
PPS	0.3
DS500	0.5
Heparin	4
HS	>50

[a] The ED_{50} values were estimated from the graphs shown in Fig. 2.

quent experiments because they were relatively strong inhibitors of PrP^{Sc} formation.

Fig. 3 shows the results of long-term treatment with glycopyranosides. Mono-4SGN, and two polymers, poly-4SGN and poly-6SGN, which reduced the level of PrP^{Sc} for two-day treatment, decreased PrP^{Sc} to undetectable level during the serial passage in the presence of the compounds. In contrast, poly-6SGlc, which did not affect the PrP^{Sc} formation in 2-day treatment, did not reduce the level of PrP^{Sc} even when used long-term.

Effect of O-sulfated glycopyranosides on the expression of PrP^C

GAGs bind to N-terminal region of PrP, which contains basic amino acid residues [18,19]. In addition, GAGs are known to accelerate PrP^C endocytosis and to reduce the total PrP^C level and cell surface expression of PrP^C [20]. These facts suggest that direct interaction of GAGs with PrP^C is involved in the inhibition of PrP^{Sc} formation, although the mechanism of inhibition remains unclear. Thus, we investigated the effect of glycopyranosides on the expression of PrP^C. Neuro2a cells were treated with test compounds at 50 μg/ml for 2 days, and expression of PrP^C was analyzed by immunoblot and IFA. The positive controls DS500 and PPS clearly reduced the total level of PrP^C (Fig. 4). Similar to the positive controls, mono-4SGN, poly-4SGN, and poly-6SGN, which inhibited PrP^{Sc} formation, significantly reduced the total PrP^C level (Fig. 4). In contrast, test compounds that did not inhibit PrP^{Sc} formation (mono-6SGN, mono-6SGlc, poly-6SGlc, and poly-GlcNAc) did not reduce the total level of PrP^C. In agreement with the immunoblot analysis, fluorescence intensities in Neuro2a cells treated with DS500, mono-4SGN, poly-4SGN, and poly-6SGN appeared to be lower than that of untreated control cells (Fig. 5). Although the total PrP^C level was reduced by the glycopyranosides, we did not observe a marked difference in localization of PrP^C.

Effect on cell growth

The inhibition of PrP^{Sc} formation by *O*-sulfated glycopyranosides suggests that they may be useful for treating prion diseases, however, the reduction of PrP^C level might

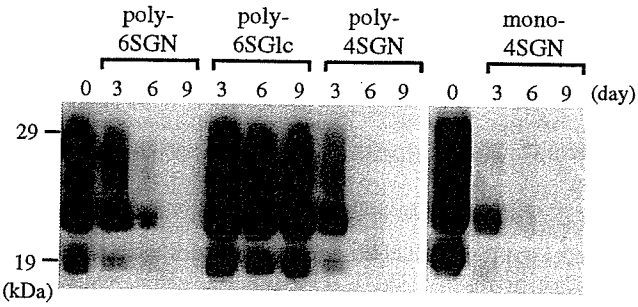

Fig. 3. Long-term effect of sulfated glycopyranosides on PrP^{Sc} biosynthesis. I3/I5-9 cells were cultured in the presence of 20 μg/ml of glycopyranosides for 3, 6, or 9 days. Day 0 indicates the untreated control.

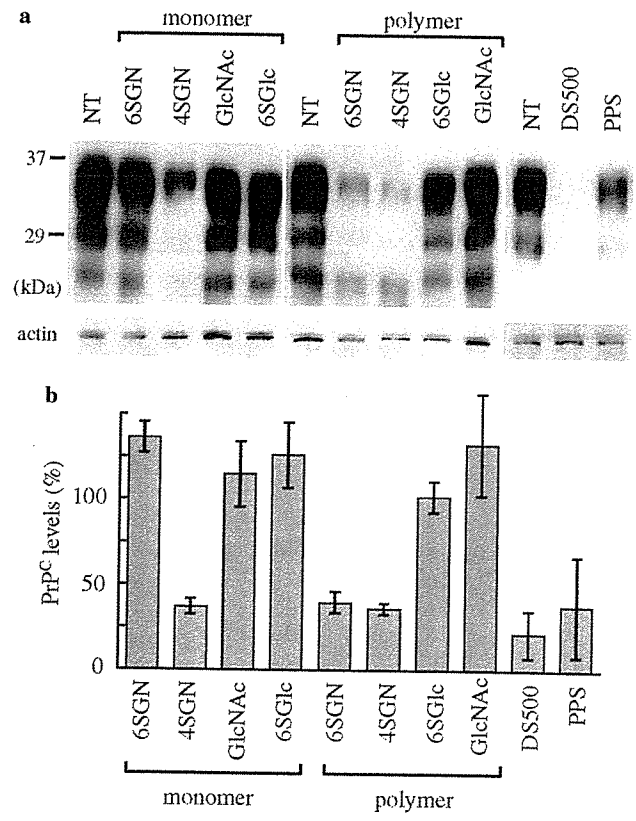

Fig. 4. Effect of sulfated glycopyranosides on PrP^C level. (a) Representative results for PrP^C detection. Neuro2a cells were treated for 2 days with various glycosides at 50 μg/ml. α-Sarcomeric actin was used as an internal loading control. PrP^C was detected with mAb 31C6. NT, untreated control. (b) Quantitative analysis of the effect of sulfated glycosides on PrP^C level. The experiment in (a) was repeated at least three times, and the graph in (b) indicates level of PrP^C relative to the untreated control.

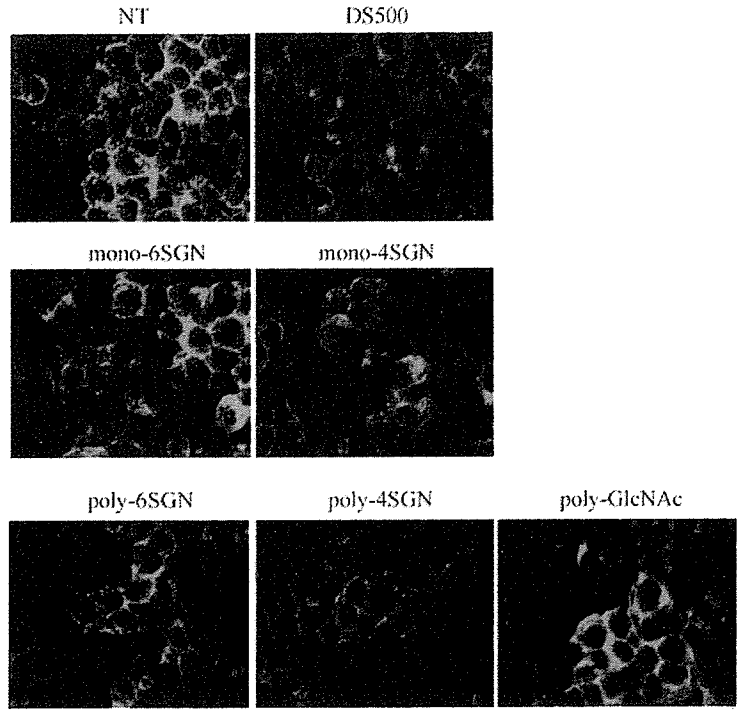

Fig. 5. Localization of PrP^C in Neuro2a cells treated with sulfated glycopyranosides. Neuro2a cells were cultured for 2 days in the presence of the indicated glycosides at 50 μg/ml. PrP^C was detected by IFA using mAb 31C6 and Alexa-488-conjugated secondary antibody. NT, untreated cells.

produce side-effects. To examine whether the synthetic glycopyranosides influence cell growth or have cytotoxicity, we performed WST-1 and lactate dehydrogenase-release assay. We found that mono-4SGN, poly-4SGN, and poly-6SGN had no effect on cell growth (Fig. 6) and were not cytotoxic (data not shown) at any of the concentrations examined.

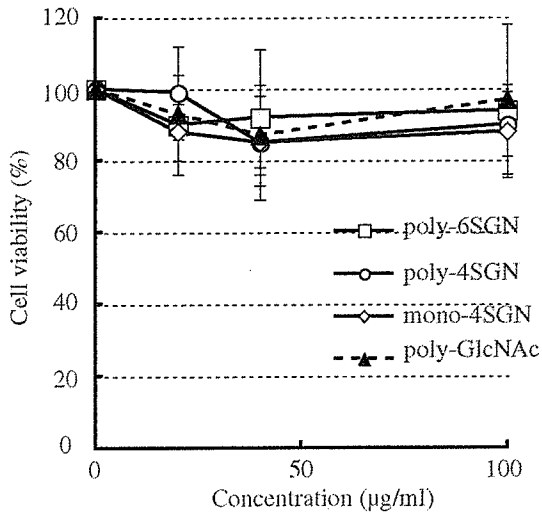

Fig. 6. Effect of sulfated glycopyranosides on cell growth. Cell growth in the presence of sulfated glycosides was determined by WST-1 assay as described in Materials and methods. Values represent means ± SD ($n = 4$) relative cell growth compared to the untreated control.

Discussion

Sulfated glycans inhibit PrP^{Sc} formation in prion-infected cells [6,7], prevent scrapie infection by peripheral challenge [2–5], and reduce the level of PrP^{Sc} in the brain of prion-infected mice [8]. The degree of sulfation appears to be one of the factors affecting anti-prion activity of sulfated glycans [21,22], although other properties, such as the chain length, repeating unit of glycans, the location of sulfate groups, and the type of glycan chains, will also be involved in the inhibition of PrP^{Sc} formation [6,23]. Selective desulfation is one way to address the SAR of sulfated GAGs [24], however, in the current studies, to examine the SAR of sulfated glycans for inhibition of PrP^{Sc} formation, we used synthetic sulfated glycopyranosides and their polymers, in which the position of sulfation was controlled by chemical and enzymatic reactions [11–13].

Among the compounds tested, mono-4SGN, poly-4SGN, and poly-6SGN inhibited PrP^{Sc} formation with ED_{50} below 20 μg/ml. This suggests that a combination of an *N*-acetyl group at *C*-2 and an *O*-sulfate group at either *O*-4 or *O*-6 on glucopyranoside is important for the inhibition of PrP^{Sc} formation. In fact, the monomeric and polymeric forms of GlcNAc and 6SGlc did not inhibit PrP^{Sc} formation, emphasizing the importance of both the *N*-acetyl group at *C*-2 and the *O*-sulfate group at *O*-6 in the inhibition by 6SGN. However, mono-6SGN did not inhibit PrP^{Sc} formation, suggesting that polyvalent or cluster effects are also important for the inhibitory effect of poly-6SGN.

Heparin is a sulfated GAG that inhibits PrP^{Sc} formation in cells [6,7]. Major constituents of heparin are disaccharide units consisting of 2-*O*-sulfate-L-iduronic acid and 2-*N*-, 6-*O*-disulfate D-glucosamine, although the sulfation sites are not always sulfated [25]. Thus, the inhibitory effect of poly-6SGN suggests that an *O*-sulfate at *O*-6 and an *N*-acetyl group at *C*-2 participate in the anti-prion effect of heparin. Preliminary experiments showed that 2-*N*-, 6-*O*-disulfate glucosamine and its polymer inhibited PrP^{Sc} formation (data not shown), supporting the role of an *N*- or *O*-sulfate group at *C*-2 and *C*-6 in the anti-prion effect of heparin.

Here, we showed that mono-4SGN inhibited PrP^{Sc} formation with an ED_{50} below 20 μg/ml. To our knowledge, this is the first report that a monomeric glycoside antagonizes PrP^{Sc} formation. The GlcNAc did not inhibit PrP^{Sc} formation, suggesting a combination of *O*-sulfate group at *C*-4 and *N*-acetyl group at *C*-2 is important for the effect of mono-4SGN. However, further analyses of other glucopyranosides such as 4SGlc will be required to address the importance of *O*-sulfate group at *C*-4 more precisely. Although poly-4SGN was more effective than the monomer, monomeric glycosides have an advantage with respect to understanding the SAR of GAGs and for the development of new therapeutic compounds against prion diseases. The purpose of this study was mainly focused to analyze the effect of sulfated glucopyranosides on PrP^{Sc} formation as constituents of GAGs. However, we also found that mono-6SGal showed weak inhibitory effect ($ED_{50} = 31$ μg/ml) and the effect was enhanced in its polymer form ($ED_{50} = 21$ μg/ml). Thus this finding will prompt to analyze the effect of other sulfated galactopyranosides.

Treatment of cells with sulfated glycans such as PPS and DS500 stimulated endocytosis of PrP^{C} and reduced the total and cell surface level of PrP^{C} [16,20]. Reduction of the amount of PrP^{C}, i.e., reduction of the amount of substrate available for PrP^{Sc} biosynthesis, may be linked to the inhibition of PrP^{Sc} formation. In this study, mono-4SGN, poly-4SGN, and poly-6SGN reduced the PrP^{C} level to about 50% of that in untreated cells, suggesting that the mechanism of the inhibition is similar to that of PPS and DS500. In contrast, a chemically modified dextran, heparan mimetics HM 2062, was reported to inhibit PrP^{Sc} formation without altering the level of PrP^{C} [21,22]. Thus, there may be several mechanisms for the inhibition of PrP^{Sc} formation by sulfated glycans. HS binds to PrP^{C} possibly via the N-terminal portion of PrP^{C} and this interaction is enhanced by Cu(II) [18,19]. The interaction between PrP^{C} and HS is thought to be involved in the biosynthesis of PrP^{C} and possibly in the conversion of PrP^{C} into PrP^{Sc} [26,27]. Thus, exogenous sulfated glycans may compete with endogenous GAG in binding to PrP^{C} or other molecules such as laminin receptor precursor/laminin receptor. As a consequence, blocking the interaction of PrP^{C} with an endogenous GAG may inhibit PrP^{Sc} formation.

This study provided new information on the SAR of GAGs in the inhibition of PrP^{Sc} formation. In addition, the inhibition of PrP^{Sc} formation by the monoglycoside, mono-4SGN, showed that studies of synthetic sulfated glycosides can aid in the development of compounds for treating prion diseases. Although it is unlikely that sulfated polyanions can pass through blood-brain barrier (BBB), small molecule such as mono-glycosides may be able to pass. The aglycons of glycopyranosides, *p*NP residue, can be modified to hydrophobic moieties [28]. Such modification may facilitate the delivery of the glycopyranosides to the brain through BBB. Further studies using synthetic sulfated glycosides may provide lead structures for the development of new compounds for the treatment of prion diseases.

Acknowledgments

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Identification of pH-sensitive regions in the mouse prion by the cysteine-scanning spin-labeling ESR technique

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Abstract

We analyzed the pH-induced mobility changes in moPrP^C α-helix and β-sheets by cysteine-scanning site-directed spin labeling (SDSL) with ESR. Nine amino acid residues of α-helix1 (H1, codon 143–151), four amino acid residues of β-sheet1 (S1, codon 127–130), and four amino acid residues of β-sheet2 (S2, codon 160–163) were substituted for by cysteine residues. These recombinant mouse PrP^C (moPrP^C) mutants were reacted with a methane thiosulfonate sulfhydryl-specific spin labeling reagent (MTSSL). The $1/\delta H$ of the central (^{14}N hyperfine) component ($M_I = 0$) in the ESR spectrum of spin-labeled moPrP^C was measured as a mobility parameter of nitroxide residues (R1). The mobilities of E145R1 and Y149R1 at pH 7.4, which was identified as a tertiary contact site by a previous NMR study of moPrP, were lower than those of D143R1, R147R1, and R150R1 reported on the helix surface. Thus, the mobility in the H1 region in the neutral solution was observed with the periodicity associated with a helical structure. On the other hand, the values in the S2 region, known to be located in the buried side, were lower than those in the S1 region located in the surface side. These results indicated that the mobility parameter of the nitroxide label was well correlated with the 3D structure of moPrP. Furthermore, the present study clearly demonstrated three pH-sensitive sites in moPrP, i.e., (1) the N-terminal tertiary contact site of H1, (2) the C-terminal end of H1, and (3) the S2 region. In particular, among these pH-sensitive sites, the N-terminal tertiary contact region of H1 was found to be the most pH-sensitive one and was easily converted to a flexible structure by a slight decrease of pH in the solution. These data provided molecular evidence to explain the cellular mechanism for conversion from PrP^C to PrP^{Sc} in acidic organelles such as the endosome.

Keywords: SDSL; ESR; Prion; Domain mobility; pH-sensitive region

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of fatal neurodegenerative disorders including Creutzfeldt–Jacob disease, Gerstmann–Sträusler–Scheinker syndrome, fatal familial insomnia, and kuru in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle [1,2]. According to the "prion-only hypothesis" [1,3,4], the abnormal (scrapie-like and β-sheet-rich) form of prion protein (PrP^{Sc}) converted from the normal cellular prion protein (PrP^C) is recognized as the only pathogenic component of TSEs. Mammalian PrP^C is a ubiquitous glycoprotein attached to the plasma membrane via a glycosyl phosphatidylinositol (GPI) anchor [1]. As illustrated in Fig. 1A, mouse PrP (moPrP) consists of 208 amino acids (residues 23–231). The carboxy-terminal domain of moPrP (121–231) is defined as a tertiary structure and contains three α-helices

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