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Bone marrow stroma cells are susceptible to prion infection

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ABSTRACT

Abnormal protease-resistant prion protein (PrP-res) is the only surrogate biochemical marker for prion diseases, and a sensitive technique to detect PrP-res in blood or tissues is urgently needed. Primary cultured bone marrow stromal cells (MSCs) expressed PrP and were capable of supporting stable human prion infection. Using a mouse-adapted BSE strain, we demonstrated that PrP-res can be detected in expanded MSCs. We then analyzed the bone marrow cells collected at autopsy from two individuals with sporadic Creutzfeldt-Jakob disease (CJD), and, in both cases, cultured MSCs were positive for PrP-res. These data would suggest that ex vivo MSC expansion accompanied by PrP-res analysis could be a helpful tool in the definitive diagnosis of prion disease at an earlier stage in the disease process than is currently possible, and with considerably less distress to the patient.

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Creutzfeldt-Jakob disease (CJD) in man is thought to be caused by an "infectious protein particle", termed prion [1]. Accumulation of the disease-associated form of prion protein (PrP^{Sc}) and infectivity are seen mainly in the central nervous system (CNS), but are not limited to the CNS. Infectivity in the blood of sporadic CJD patients and experimentally infected animals has been reported [2], and recently it has been shown that variant CJD (vCJD) can be transmitted by transfusion [3,4]. The problem, however, is that detection of PrP^{Sc} in blood is extremely difficult and no reliable test exists [5]. For this reason, we instead focused on bone marrow stroma cells (MSCs) [6,7], which possess multipotential stem cell-like characteristics, and investigated whether or not they were susceptible to TSE agents. Ex vivo cultured MSCs expressed PrP^{C} and were susceptible to a CJD agent. In addition, we were able to detect PrP^{Sc} in MSCs isolated from both infected animals and sporadic CJD patients. These results suggest that bone marrow biopsy followed by ex vivo expansion of MSCs could form the basis of a new diagnostic test for TSEs.

Materials and methods

Isolation and culture of MSCs. Adult male Wistar rats, 8 weeks old, were killed and the femurs and tibias were dissected out. Isolation of the bone marrow was performed according to the method described by Azizi et al. [7]. The ends of the bones were cut and the marrow was extruded with 5 ml of alpha-MEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml kanamycin, or 100 U/ml penicillin and 100 μg/ml streptomycin. The bone marrow cells were incubated at 37 °C with 95% humidity and 5% CO_2 for 48 h, and the non-adherent cells removed by replacing the medium. Adherent cells were subcultured several times, and used for the transmission studies. The cells were subjected to a neuronal differentiation study to confirm that they were stromal cells (MSC), according to Dezawa's method [8,9]. Additionally, bone marrow stromal cells isolated from mice, hamsters and cows were cultured in the same way as for rat MSCs. Normal human MSCs were purchased from Cambrex Bio Science Walkersville, Inc. In the case of CJD patients, informed consent was obtained from the patient's family, and the investigation protocol was approved by the Ethics Committee of Nagasaki University Hospital (ID 06012755).

Animal TSE models. To establish a rat TSE model, 10% (w/w) homogenates of a brain taken from a Gerstmann-Sträussler-Scheinker syndrome (GSS) patient carrying the P102L mutation in PRNP [10] were prepared with sterile PBS. We first inoculated the homogenate into Wistar rats (3 weeks old) and NZW mice (4 weeks old). Although all the rats ($n = 6$) remained free of any neurological signs and were healthy until 2 years post inoculation, the NZW mice developed typical mouse TSE after around 230 days. The mouse brain homogenate was then inoculated into Wistar rats, and those rats developed disease at about 330 days. Accumulation of PrP^{Sc} in the affected brains was confirmed by Western blotting (data not shown), and by histology, in which common characteristics of prion disease such as spongiform change, neuronal loss, and gliosis were observed, and accumulated PrP^{Sc} was stained diffusely

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by 6H4 antibody. The agent isolated was designated Nagasaki University-1 isolate (Nu-1).

Mouse BSE model. Primary infection of a BSE agent isolated from a cow in the UK was done using RIII mice (killed at 412 days) and the affected brains were kindly provided to us by Dr. Yokoyama (Tsukuba). We then passed this mouse-adapted BSE into ddY mice twice. Mice intracerebrally inoculated with brain homogenate all developed disease at around 150 days, with infectivity in the brain reaching around $10^{8.9}$ LD_{50}/g. At the third passage, 100 μl of a 10% brain homogenate were inoculated into the peritoneal cavity, and samples of bone marrow, spleen, and brain were collected every 4 weeks. Infected mice showed abnormal gait and other neurological signs at around 20 weeks post inoculation and died at 28 wpi. In some experiments, mouse-adapted GSS agent, Fukuoka-1 strain [11], was also inoculated into ddY mice.

Ex vivo infection (Prion infection to the MSC). Normal rat MSCs (passage 5) growing in a T25 flask were exposed to 0.2% of Nu-1 rat brain homogenate for 24 h, after which an equal volume of fresh medium was added and the incubation was continued for another 48 h. Then, after washing the cells several times with PBS, we split the cells at a ratio of 1:3. At every passage, confluent cells were lysed and subjected to Western blotting for PrP^{Sc} as described below.

PrP^{Sc} detection. Brain, spleen, and lymph node were homogenized at 10% in phosphate buffered saline. Total proteins were extracted by mixing with 1% Triton X-100/DOC buffer (1% Triton X-100, 1% Deoxycholic acid, 300 mM NaCl, 50 mM Tris–HCl pH 7.5) and the mixture was centrifuged at 500*g* at 4 °C for 15 min to remove cell debris. The supernatants were digested with Proteinase-K (20 μg/mg protein, 37 °C, 30 min) and mixed with sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris–HCl, pH 6.8, containing 5% glycerol, 1.6% SDS, and 100 mM dithiothreitol) and boiled for 10 min. Confluent cell cultures were lysed for 30 min at 4 °C in 0.5% Triton X-100/DOC buffer (0.5% Triton X-100, 0.5% Deoxycholic acid, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5). The supernatant was collected after a short centrifugation at 10,000*g*, and the total protein concentration was measured using the BCA protein assay (Pierce). The protein concentration was adjusted to 1.0 mg/ml, and digested with proteinase K (PK) (40 μg/mg protein, 37 °C, 30 min), and then PK-resistant and insoluble PrP was concentrated by centrifugation at 19,000*g* for 45 min at 4 °C. The pellet was dissolved in 1× SDS loading buffer and boiled for 10 min.

In the cell samples as well as the tissues samples, PrP^{Sc} was detected by Western blotting. SDS–polyacrylamide gel electrophoresis (PAGE) was performed using 1.5 mm, 12% acrylamide gels. The proteins were transferred onto an Immobilon-P membrane (Millipore) in transfer buffer containing 20% methanol, and the membrane was blocked with 5% nonfat dry milk in TBST (10 mM Tris–HCl pH 7.8, 100 mM NaCl, 0.1% Tween 20) for 60 min at room temperature. Blocked membranes were reacted with anti-PrP antibodies diluted 1:1000 in 1% nonfat dry milk/TBST overnight at 4 °C. The membranes were then washed 3 times for 5 min in TBST, reacted with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted 1:5000 in 1% nonfat dry milk/TBST for 60 min at room temperature and washed again 5 times for 5 min in TBST. Immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech). The anti-PrP polyclonal mouse antiserum (SS#28) used has been described previously. The M20 goat antibody to C-terminal PrP peptides was purchased from Santa-Cruz Biotech (Santa Cruz, CA) [12]. Horseradish peroxidase (HRP)-conjugated anti-goat immunoglobulin G antibodies were purchased from Santa-Cruz Biotech.

Immunohistochemistry. Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned into 5 μm slices. The tissue sections were stained with hematoxylin and eosin (HE), or subjected to immunological staining of PrP, using the hydrolytic autoclaving and formic acid method prior to incubating with primary antibodies such as SAF32 (SPI bio, France) and 6H4 (Prionics AG, Switzerland) overnight, and then the sections were reacted with envision polymer horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G antibodies (vectastain ABS Kit, Dako Cytomation) for 60 min. Immunostaining was visualized by 3, 3′-diaminobenzidine chromogen via a horseradish peroxidase reaction.

Results and discussion

MSCs have unique characteristics. They grow well ex vivo without transformation and are multipotential progenitor cells which can be used for auto-cell transplant therapy[8]. We isolated and subcultured bone marrow cells, and were able to obtain relatively uniform cell types from human, rat and bovine samples, but not from those of mouse and hamster. (Fig. 1A). The rat bone marrow cells grew for more than 2 years (100 passages), but the human cells stopped dividing at about 15 passages, and the bovine, mouse and hamster cells continued to grow for only a few passages. To confirm that the cells isolated from rat bone marrow were indeed MSCs, we tried to differentiate them into neurons, according to the method described by Dezawa et al. [9] (data not shown). To examine the expression of PrP^{C}, MSCs were analyzed by Western blotting. Anti-PrP monoclonal antibody (SAF32) raised against N-terminal octapeptide repeats of PrP could detect normal PrP in the MSCs from all species (Fig. 1B), and its expression on the cell surface was also confirmed by immunostaining (Fig. 1C).

Isolated MSCs from uninfected rat bone marrows were subjected to an ex vivo transmission study using a rat-adapted GSS strain, Nagasaki isolate (Nu-1). Primary cultured rat MSCs (P3) were incubated with 0.2% brain homogenate and passaged for more than 2 years. In the early phase, little or no PrP^{Sc} could be detected in the cultured MSCs, but with repetition of passages, the amount increased (Fig. 2) and multinuclear cells were observed. The ex vivo infected MSCs initially produced only small amounts of PrP^{Sc}, which increased with passaging, and stable PrP^{Sc} production was confirmed after 50 passages, suggesting that the infection spread only gradually. A similar observation has been reported in mouse neuronal cells [12]. To confirm that the MSC/Nu-1 cells were indeed infected with the agent, we inoculated the cell lysates (P25 and P50) intracerebrally into Wistar rats (4 weeks old). After one year, the rats all showed signs such as reflection abnormality, and Western blotting of the brains confirmed the presence of PrP^{Sc} (data not shown). These results provide evidence that the MSCs express PrP^{C} and are susceptible to TSE agents, and are capable of supporting stable infection.

To elucidate whether or not MSCs can be infected with the TSE agent in vivo, we isolated MSCs from terminally sick animals. The MSCs from Nu-1 Wistar rat at 300 dpi grew slowly compared with those from young rats, and we were able to detect PrP^{Sc} (data not shown). In addition, MSCs from terminally sick mice infected with mouse-adapted BSE or Fukuoka-1 agents were also positive for PrP^{Sc} (Fig. 3A). Next, we inoculated MSCs from four terminal BSE-infected mice into the brains of normal ddY mice (five each). After 20 weeks, all exhibited a yellowish pubic region and abnormal hind limb reflex, but after that, clinical signs worsened only slowly. After 25 weeks, we sacrificed some of the mice and were able to confirm accumulation of abnormal PrP in the spleens (Fig. 3B, arrows). This result provides evidence that MSCs in vivo do indeed carry the agent.

We next asked how soon the PrP^{Sc} could be seen in MSCs from infected mice. After intraperitoneal inoculation of 100 μl of the BSE mouse 10% brain homogenate, brains, spleens, and bone marrows were collected from 4 or 5 animals every 4 weeks. PrP^{Sc} was first

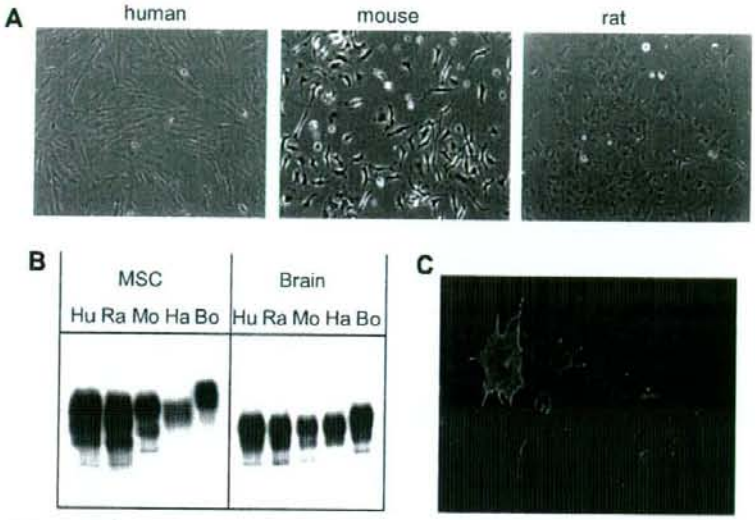

Fig. 1. Bone marrow stromal cells express PrP^C. (A) Phase-contrast pictures of human, mouse, and rat MSCs. (B) Western blotting shows PrP^C expression in these cells. All MSCs isolated from different animals were positive for PrP^C. (C) Immunostaining for PrP on rat MSCs. Typical punctuated staining was seen on the cell surface.

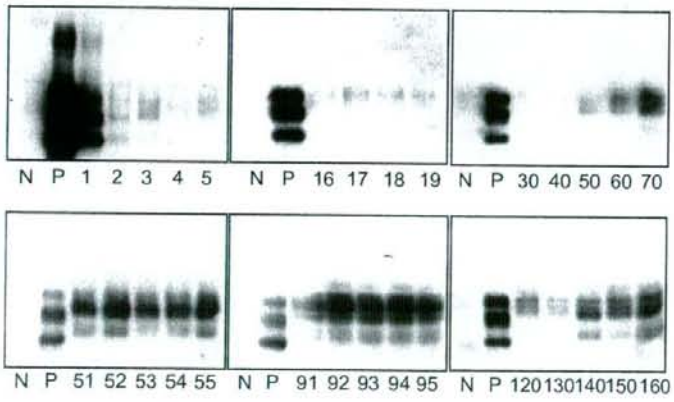

Fig. 2. Ex vivo transmission of Nu-1 agent on rat MSCs. PrP signals for the Proteinase-K resistant form of PrP (PrP^{Sc}) were seen in the culture. N: uninfected normal control. P: Nu-1 rat brain homogenate sample. Numbers indicate times of passages from infection.

detected in spleen at 4 wpi and in brains at 24 wpi. MSCs from bone marrow were isolated and expanded (passaged 3 times) in order to harvest enough cellular proteins, and showed weak PrP^{Sc} signals at 4 wpi, which disappeared at 8 wpi, and were again detected at 16–28 wpi. (Fig. 3C). This indicates that infection of the MSCs in vivo could precede the accumulation of PrP^{Sc} in brain.

PrP is expressed mainly in the brain but also in many other organs, including the lymphoreticular system tissues (LRS) [13,14]. Although the role of PrP^C in blood cells remains to be established, PrP^C is known to be expressed by hematopoietic stem cells (HSCs) [15], immature and mature T cells, B cells, monocytes and dendritic cells (DCs) [16]. CD43+ Gr-1+ granulocyte precursors in bone marrow have also been shown to express PrP [17]. When we analyzed murine bone marrow by in situ hybridization, PrP^C mRNA stained in HSCs but not MSCs (data not shown). Therefore, although we cannot exclude the possibility that MSCs start expressing PrP when the cells are cultured ex vivo, our data would suggest that MSCs could express PrP and be a long-term reservoir for TSE agents in vivo, in which case circulating white blood cells could also become infected by contact with the MSCs.

To determine whether human MSCs are also susceptible to TSE agents, we examined bone marrows obtained at autopsy from two CJD patients. The first case was a 66-year-old female who had been clinically diagnosed as having sporadic CJD. The patient had died 27 months after the onset. An autopsy was held 7 h after her death, and we collected bone marrow from the sternum. Because coagulation of the blood had already advanced, we were able to collect only an extremely small amount of bone marrow cells and it took about 2 months to grow sufficient MSCs for the experiment. One milligram of extracted protein was digested and concentrated at 10,000g for 1 h, then subjected to Western blotting for PrP^{Sc} using 3F4 antibody. A relatively weak but clear signal was seen in this patient's MSCs (Fig. 4A). The second case was a 70-year-old male who also had been diagnosed with sporadic CJD. Abnormally ele-

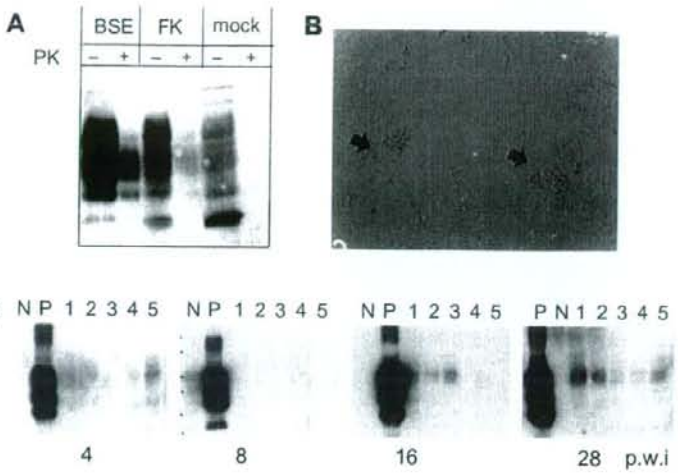

Fig. 3. PrP^{Sc} was detected in MSC culture from terminally sick animals and was infectious. (A) MSCs were isolated from mouse-BSE infected and Fukuoka-1 infected ddY mice. Both cultures were positive for PrP^{Sc}. (B) Immunostaining for PrP^{Sc} in spleen obtained from a mouse inoculated with BSE-MSCs, indicating the cells were truly carrying infectivity. (C) Time-course analysis of PrP^{Sc} in BSE-inoculated mice. MSCs were isolated from intraperitoneally infected mice and PrP^{Sc} was detected. Note that PrP^{Sc} was seen in MSCs as early as 16 wpi.

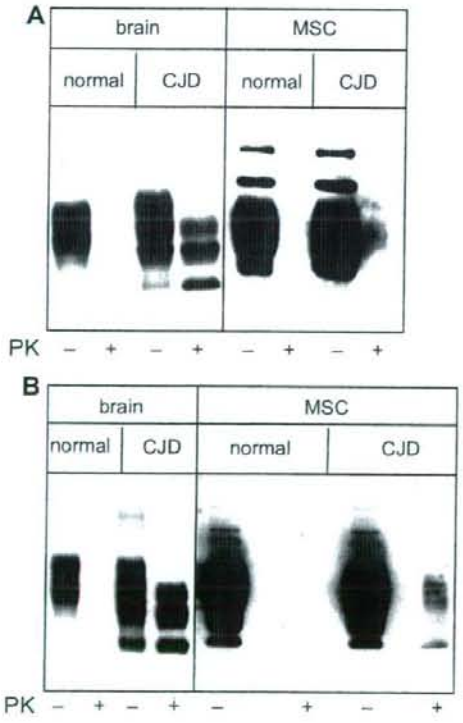

Fig. 4. (A) *Ex vivo* expanded MSCs from CJD patients were positive for PrP^{Sc}. (A) Bone marrow cells were isolated at autopsy of a sporadic CJD patient. After expansion of MSCs for a month, Western blotting for human PrP could detect PrP^{Sc} in the cell lysate. (B) MSCs from another sporadic CJD patient. Bone marrow was obtained immediately after death. PrP^{Sc} was seen in the culture even at P1. PK: Proteinase-K digested sample.

vated signals in the brain had been observed during screening with diffusion NMR, at which time the patient was free of symptoms. A few months later, he presented with mild dementia and neurological abnormalities. As the clinical manifestations progressed, CSF analysis showed that 14-3-3 was elevated and CJD was diagnosed. The patient died about a year after the onset. This time, we were able to collect bone marrow immediately after death. A good amount of adherent cells was observed in the primary culture dishes, and after expanding them we were able to recover the cellular proteins and perform Western blotting analysis. PrP^{Sc} was detected even in the P1 cell lysate (Fig. 4B). These findings suggest that the ex vivo expansion of MSCs could be an alternative diagnostic method for CJD.

Hadlow et al. were the first to report that infectivity could be reached at 10^5 LD_{50} in the bone marrow (whole femur) of Swiss mice infected with a mouse-adapted scrapie strain, Chandler isolate [18,19]. Others, however, subsequently failed to detect PrP^{Sc} in bone marrow by Western Blotting [20]. Brown et al. inoculated the bone marrow from CJD patients into two normal primates, neither of which went on to develop TSE [21]. These results might mean that there was little infectivity in the samples used, or accumulation of PrP^{Sc} and infectivity in bone marrow might depend on either the strain of agent or the host species. How real the risk of infectivity is in the bone marrow of CJD patients remains to be assessed using sensitive models. At this point, it is too early to state that CJD could be transmitted during bone marrow transplantation, as no accidental transmission has been reported to date.

Because the clinical features of CJD vary, definitive diagnosis relies on typical brain pathology and the post mortem detection of the pathological form of prion protein in the brain tissue. Protein analysis of the cerebrospinal fluid (CSF) and diffusion NMR are useful tools for differential diagnosis of neurodegenerative disorders and contribute to the clinical diagnosis of typical CJD [22]. Recently, a method for the amplification of abnormal PrP in vitro (PMCA) from biological samples has been developed and has great potential for application to clinical practice [23,24], but it still remains to be approved for use on human blood samples. The histological examination of brain biopsy specimens is currently the only

way to confirm the clinical diagnosis of TSE before death. Bone marrow biopsy could thus lead to an early definitive diagnosis, enhancing the possibility of successful treatment.

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Commentary & View

Antagonistic roles of the N-terminal domain of prion protein to doppel

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Key words: prion protein, doppel, neurotoxic signal, neurodegeneration, neuroprotection, prion disease

Prion protein (PrP)-like molecule, doppel (Dpl), is neurotoxic in mice, causing Purkinje cell degeneration. In contrast, PrP antagonizes Dpl in trans, rescuing mice from Purkinje cell death. We have previously shown that PrP with deletion of the N-terminal residues 23–88 failed to neutralize Dpl in mice, indicating that the N-terminal region, particularly that including residues 23–88, may have trans-protective activity against Dpl. Interestingly, PrP with deletion elongated to residues 121 or 134 in the N-terminal region was shown to be similarly neurotoxic to Dpl, indicating that the PrP C-terminal region may have toxicity which is normally prevented by the N-terminal domain in cis. We recently investigated further roles for the N-terminal region of PrP in antagonistic interactions with Dpl by producing three different types of transgenic mice. These mice expressed PrP with deletion of residues 25–50 or 51–90, or a fusion protein of the N-terminal region of PrP with Dpl. Here, we discuss a possible model for the antagonistic interaction between PrP and Dpl.

The normal prion protein, termed PrP^C, is a membrane glycoprotein tethered to the outer cell surface via a glycosylphosphatidylinositol (GPI) anchor moiety.[1,2] It is ubiquitously expressed in neuronal and non-neuronal tissues, with highest expression in the central nervous system, particularly in neurons.[3] The physiological function of PrP^C remains elusive. We and others have shown that PrP^C functionally antagonizes doppel (Dpl), a PrP-like GPI-anchored protein with ~23% identity in amino acid composition to PrP, protecting Dpl-induced neurotoxicity in mice.[4-7] Dpl is encoded on *Prnd* located downstream of the PrP gene (*Prnp*) and expressed in the testis, heart, kidney and spleen of wild-type mice but not in the brain where PrP^C is actively expressed.[4,5,8] However, when ectopically expressed in brains, particularly in cerebellar Purkinje cells, Dpl exerts a neurotoxic activity, causing ataxia and Purkinje cell degeneration in Ngsk, Rcm0 and Zrch II lines of mice devoid of PrP^C ($Prnp^{0/0}$).[4,9,10] In these mice, Dpl was abnormally controlled by the upstream *Prnp* promoter.[4,5] This is due to targeted deletion of part of *Prnp* including a splicing acceptor of exon 3.[11] Pre-mRNA starting from the residual exon1/2 of *Prnp* was abnormally elongated until the end of *Prnd* and then intergenically spliced between the residual *Prnp* exons 1/2 and the *Prnd* coding exons.[4,5] As a result, Dpl was ectopically expressed under the control of the *Prnp* promoter in the brain, particularly in neurons including Purkinje cells.[4,5] In contrast, in other $Prnp^{0/0}$ lines, such as Zrch I and Npu, the splicing acceptor was intact, resulting in normal Purkinje cells without ectopic expression of Dpl in the brain.[4]

The molecular mechanism of the antagonistic interaction between PrP^C and Dpl remains unknown. We recently showed that the N-terminal half of PrP^C includes elements that might mediate cis or trans protection against Dpl in mice, ameliorating Purkinje cell degeneration.[12] We also showed that the octapeptide repeat (OR) region in the N-terminal domain is dispensable for PrP^C to neutralize Dpl neurotoxicity in mice.[12] Here, possible molecular mechanisms for the antagonism between PrP^C and Dpl will be discussed.

Lack of a cis-Protective Element Renders PrP and Dpl Neurotoxic

PrP^C largely comprises of two domains, the N-terminal and C-terminal domains (Fig. 1A). The N-terminal domain is highly flexible, lacking identifiable secondary structures.[13] This domain includes the OR region, which is unique to all PrP molecules.[2] In contrast, the C-terminal domain forms a globular structure with three α-strands and two short β-strands.[13] Interestingly, PrP with the N-terminal residues 32–121 or 32–134 deleted, termed PrPΔ32-121 and PrPΔ32-134 (Constructs 1 and 2 in Fig. 1B), respectively, was shown to be neurotoxic.[14] This caused ataxia and cerebellar neurodegeneration, including granule or Purkinje cell death in Zrch I $Prnp^{0/0}$ mice.[14,15] These results suggest that PrP^C is potentially neurotoxic via the C-terminal domain but under normal conditions the neurotoxicity of the C-terminal domain may be masked by the N-terminal domain.

Dpl is a homologue of the C-terminal globular domain of PrP^C (Fig. 1A).[16] However, Dpl lacks the amino acid sequences corresponding to the N-terminal half of PrP^C (Fig. 1A). It is therefore conceivable that the neurotoxicity of Dpl might be due to lack of the corresponding N-terminal part of PrP^C. Consistent with this, we recently showed that PrPN-Dpl (Construct 3 in Fig. 1B), a fusion protein of the N-terminal residues 1–124 of PrP^C and the residues 58–179 of Dpl, was itself non-toxic in mice.[12] It induced neither ataxia nor Purkinje cell degeneration in Zrch I $Prnp^{0/0}$ mice, even when transgenically expressed in the brain under the control of the *Prnp* promoter.[12] Constructs 1 and 2 cover most of

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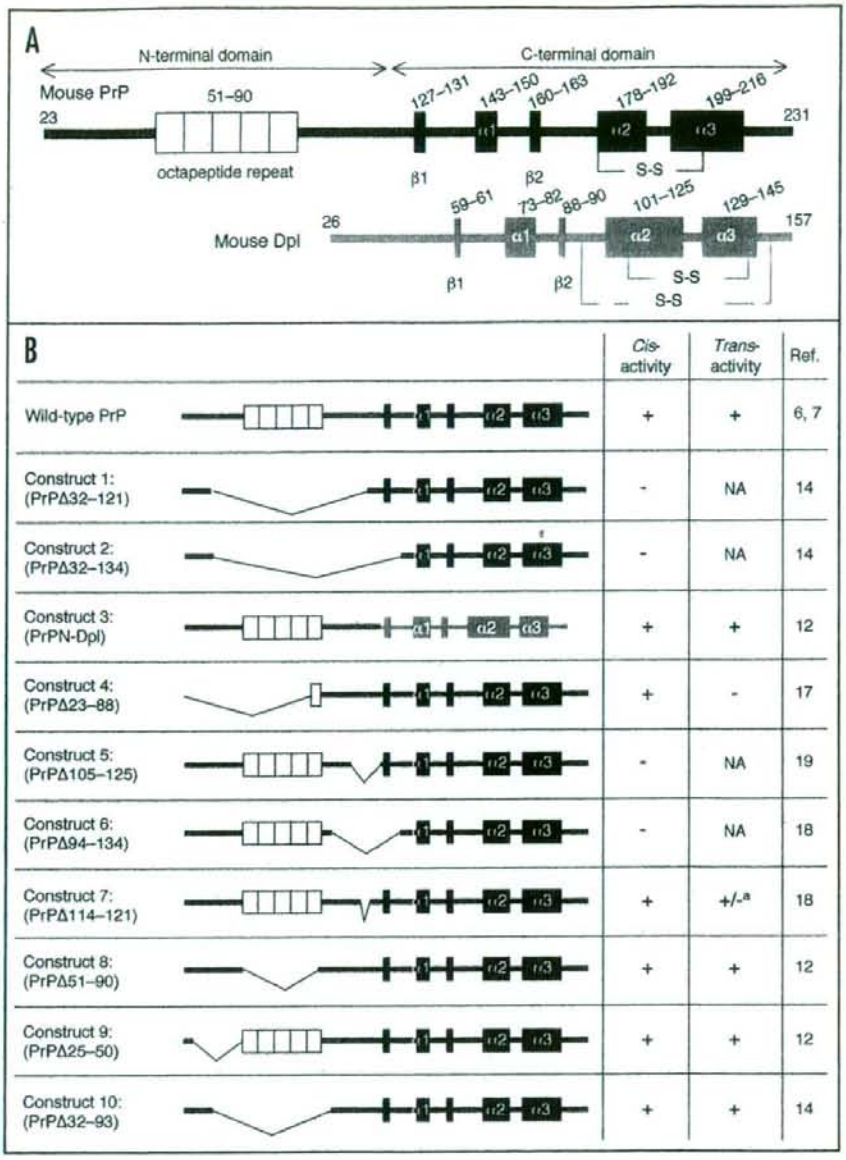

Figure 1. (A) Schemes of wild-type mouse PrP and Dpl. Mouse PrP is first translated as a precursor protein consisting of 254 amino acids. The N-terminal 22 and C-terminal 23 hydrophobic amino acids are removed as a signal peptide and a GPI-anchor signal sequence, respectively. The N-terminal half of PrP^C is highly flexible and lacks identifiable secondary structure. The octapeptide repeat (OR) region, comprising five copies of a P(H/Q)GGG(G)WGQ octapeptide sequence, is located in the N-terminal domain. The OR region is thought to mediate anti-oxidative activity by binding to Cu^{2+} via histidine residues. However, the exact function of this region remains to be elucidated. The C-terminal half of PrP^C forms a globular structure with three α-helices (α1-3) and two short anti-parallel β-strands (β1, β2). The second and third helices are linked by a disulfide bond (-S-S-). The precursor protein of Dpl consists of 179 amino acids. The N-terminal 25 and C-terminal 22 hydrophobic residues may be removed as signal peptide and GPI-anchor signals, respectively. Dpl is a structural homologue of the C-terminal globular domain of PrP^C, sharing ~23% identical amino acids and is composed of three α-helices (α1-3) and two short anti-parallel β-strands (β1, β2). Two disulfide bonds (-S-S-) are formed. However, Dpl lacks the corresponding N-terminal part of PrP^C. (B) Structural schemes of PrPs with deletion of various regions and PrPN-Dpl, the fusion protein composed of the N-terminal region of PrP with Dpl, with their cis- and trans-protective activity against Dpl or toxic PrPs are shown. a: Construct 7 is itself non-toxic. However, it has different affects on neurotoxic Constructs 2 and 6: It enhances the toxicity of Construct 6 but diminishes that of Construct 2. NA: data are not available.

the Dpl-homologous C-terminal part of PrP. These observations strongly suggest that Dpl might undergo the same or very similar molecular processes as toxic PrP molecules do to perform their neurotoxicity in mice.

In contrast to the Constructs 1 and 2, PrPΔ23-88 (Construct 4 in Fig. 1B) is non-toxic in mice.[17] This indicates that the central region including residues 89–121, which are deleted in the toxic Constructs 1 and 2 but intact in the non-toxic Construct 4, may include an element(s) that mediates the cis-protection against the neurotoxic C-terminal domain. Indeed, PrP with deletion of the central resides 105–125 or 94–134 (Constructs 5 and 6 in Fig. 1B) was shown to be neurotoxic, causing cerebellar degeneration or demyelination in mice.[18,19] However, no neurotoxicity was detected for PrP with deletion of only eight amino acids (residues 114–121) in the central region (Construct 7 in Fig. 1B).[18] These results suggest that the cis-protective activity of the central region might be regulated in a highly integrated way, which might be impaired by deletion of a large part of the region rather than any specific amino acids or small areas.

Trans-Protection by PrP Against Dpl

Trans-protective activity of various PrP constructs against Dpl or the toxic truncated PrPs is summarized in Figure 1B. Wild-type PrP^C has the potential to abrogate Dpl neurotoxicity in trans. The ataxia and Purkinje cell degeneration, which were induced by transgenic expression of Dpl in the brain, could be attenuated in mice carrying the wild-type but not the knockout genetic background

for *Prnp*.[6,7] We previously showed that Construct 4, which lacks the N-terminal residues 23–88, completely lost the ability to rescue an ataxic Ngsk line of *Prnp*$^{0/0}$ mice from Dpl-induced Purkinje cell degeneration.[17] We also recently showed that Construct 3 in which the PrP N terminal region (residues 23–124) was fused to Dpl (residues 58–179) mitigated the neurotoxicity of transgenically expressed wild-type Dpl in mice, prolonging the times to the onset of ataxia and Purkinje cell degeneration.[12] These results indicate that the N-terminal domain, particularly that encompassing residues 23–88, might include an element(s) that mediates the antagonistic function of PrP^{C} against Dpl in trans. However, the trans-protective element might require cis-protective activity to function, because the neurotoxic Constructs 5 and 6 include the trans-elements but not the cis-element.[18,19]

Residues 23–88 cover the entire pre-OR and almost the entire OR except for two amino acids (residues 89 and 90). We recently investigated the role of the OR and the pre-OR in the trans-neuroprotection of PrP^{C} against Dpl by producing transgenic mice expressing Constructs 8 or 9.[12] They expressed PrP with deletion of the entire OR (residues 51–90) or most of the pre-OR (residues 25–50) except for residues 23 and 24.[12] Complete rescue from ataxia and Purkinje cell degeneration was detected in mice co-expressing the OR-lacking Construct 8 and Dpl in the absence of wild-type PrP^{C},[12] clearly indicating that the OR is dispensable for PrP^{C} protection against Dpl-neurotoxicity in trans. The pre-OR-lacking Construct 9 also blocked Dpl-neurotoxicity in mice in a manner dependent on its expression level, prolonging the onset of ataxia and Purkinje cell death.[12] Shmerling et al. reported that the cerebellar granule cell death induced by the neurotoxic Construct 2 in Zrch I *Prnp*$^{0/0}$ mice could be abrogated by Construct 10.[14] Construct 10 lacks the entire OR and part of the pre-OR. These findings indicate that the OR and part of the pre-OR are also unnecessary for PrP^{C} to antagonize the neurotoxicity of truncated PrPs in trans.

Two amino acids (residues 23 and 24) of the pre-OR are commonly intact in the trans-protective molecules, including wild-type PrP^{C} and Constructs 3 as well as 8–10, but not in the non-protective Construct 4. It is therefore possible that these two residues are important for the trans-neuroprotection of PrP^{C} against Dpl or the toxic truncated PrPs. Interestingly, the two amino acids are followed by residues starting from 51 in Construct 9, generating a new N-terminal sequence (KKPQGGTWG), which is very similar to the N-terminal 9 residues (KKRPKPGGW) of wild-type PrP^{C}. Six out of nine of these amino acids are identical. It is thus possible that this newly generated N-terminal sequence might mimic the function of wild-type N-terminal 9 residues. This N-terminal sequence also remains intact in the other protective Constructs 3, 8 and 10. This therefore suggests that rather than the two amino acids, the 9 N-terminal residues may be relevant to the trans-neuroprotection of PrP^{C} against Dpl. It might be alternatively possible that the trans-neuroprotection of PrP^{C} against Dpl may be impaired only by a large deletion of the N-terminal domain, such as deletion of the residues 23–88, but not by small deletions such as deletion of part of pre-OR and/or OR.

Possible Mechanism of Antagonistic Interaction between PrP and Dpl

The exact mechanism by which PrP^{C} antagonizes Dpl, preventing Purkinje cell degeneration, remains elusive. Accumulating evidence indicates that PrP^{C} might function as a neuroprotective molecule by exerting anti-apoptotic activities. Indeed, we and others showed that *Prnp*$^{0/0}$ mice were highly sensitive to ischemic or traumatic brain damage, developing more severe apoptotic neuronal cell death than in wild-type mice.[20-23] Moreover, it was reported that hippocampal neuronal cell lines established from *Prnp*$^{0/0}$ mice easily succumbed to apoptosis after serum withdrawal, and that expression of either PrP^{C} or the anti-apoptotic molecule Bcl-2 rescued cell lines from the apoptosis.[24] PrP^{C} also prevented Bax-induced apoptosis in human primary neurons.[25] Interestingly, PrP lacking OR failed to rescue the cells from the apoptosis, which was induced by serum withdrawal or Bax, and Zrch I *Prnp*$^{0/0}$ mice from ischemic brain damage.[25-27] This indicates that the OR is essential for the neuroprotective activity of PrP^{C}. However, we demonstrated that the OR is dispensable for PrP^{C} to antagonize Dpl in mice.[12] Shmerling et al. also showed that the OR is unnecessary for PrP^{C} to antagonize the neurotoxicity of truncated PrPs.[14] These indicate that the neuroprotective activity of PrP^{C}, especially mediated via the OR, may not be required for the antagonistic function of PrP^{C} against Dpl.

Some models postulate that PrP^{C} interacts with an as yet unidentified transmembrane molecule that transmits a neuroprotective or cell survival signal.[14,18] Dpl and the toxic truncated PrPs could bind to the molecule, but generate no signal due to lack of the N-terminal domain, resulting in neuronal cell death. According to these models, PrP molecules that fail to generate the signal should be toxic, like Dpl or the toxic truncated PrPs. However, inconsistent with this, we previously showed that Construct 4 lacking residues 23–88, failed to elicit the antagonistic signal against Dpl but was itself non-toxic to neurons.[17]

Wong et al. reported that Dpl-expressing Rcm0 *Prnp*$^{0/0}$ mice produced oxidative stress of radical oxygen species or nitric oxide in their brains much more than non-expressing Npu *Prnp*$^{0/0}$ mice.[28] This therefore suggested that Dpl may actively produce the neurotoxic signal, causing neuronal cell degeneration. Dpl is a GPI-anchored membrane glycoprotein, thus requiring interaction with a transmembrane molecule to transmit the signal (Fig. 2A). The toxic PrP molecules may interact with the molecule via the Dpl-homologous C-terminal domain in the same way as Dpl, eliciting a neurotoxic signal (Fig. 2A). However, the central region may interfere with the interaction, thereby preventing the neurotoxicity of the C-terminal of PrP in cis (Fig. 2B). Rambold et al. reported that PrP with the domain spanning central residues 113–133 deleted, termed PrPΔHD, failed to form a homo-dimer, being toxic by inducing apoptosis in human neuroblastoma SH-SY5Y cells.[29] It is thus possible that the central residues may be involved in dimerization of PrP, thereby preventing the C-terminal domain of PrP from interaction with the transmembrane molecule (Fig. 2B). Alternatively, the central residues may form intra-molecular interaction with the C-terminal region of PrP, thereby inhibiting the binding between it and the transmembrane molecule (Fig. 2B). The N-terminal region which mediates the trans-protective activity, may also bind to the transmembrane molecule only when the cis-element is intact, but

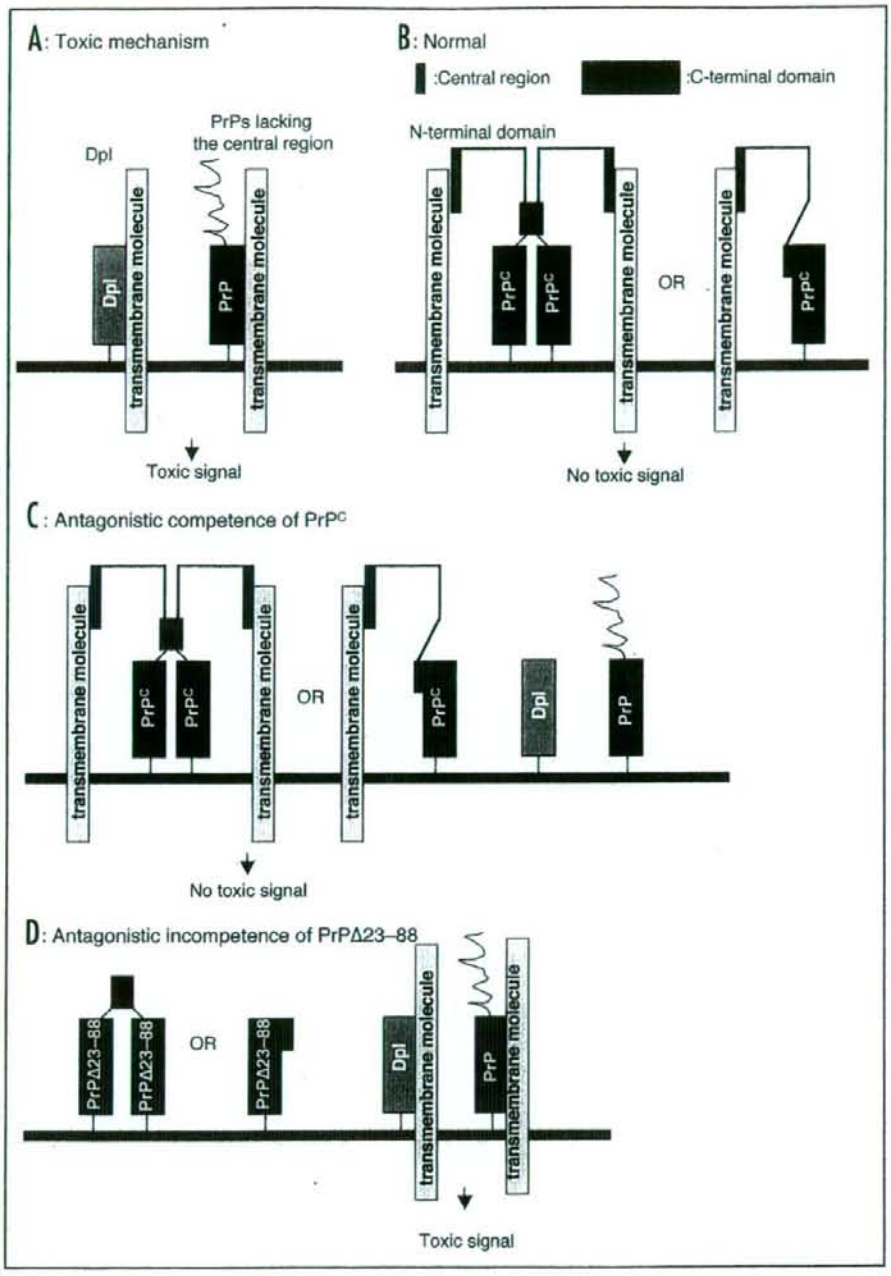

Figure 2. A possible mechanism for the antagonistic interaction of PrP^C and Dpl or the toxic PrPs. (A) Dpl binds to a putative transmembrane molecule, producing a toxic signal. Toxic PrPs with deletion of the central region, such as Constructs 1, 2, 5 and 6, bind to the transmembrane molecule via the Dpl-homologous C-terminal area in the same way as Dpl, eliciting a similar toxic signal. (B) Under normal conditions, wild-type PrP^C binds to the transmembrane molecule via the N-terminal region but not its C-terminal region because it forms either a homo-dimer linked via the central region or a monomer with the central region interacting with part of the C-terminal domain. The N-terminal region acquires binding affinity to the molecule only when the central region is intact. However, this type of interaction produces no toxic signal. (C) PrPs with part of the N-terminal region and with the central region both intact, such as trans-protective PrPs, have a higher affinity for the transmembrane molecule than Dpl or the toxic PrPs, resulting in trans-protection against Dpl and the toxic PrPs. (D) Construct 4 (PrPΔ23-88) still has potential to form a homo-dimer due to the residual central region or a monomer with the residual central region masking part of the C-terminal region, similarly to wild-type PrP^C. Therefore, Construct 4 cannot form a complex with the transmembrane molecule via the C-terminal region, generating no toxic signal. In addition, by lacking part of the N-terminal domain, Construct 4 has no affinity for the transmembrane molecule, losing trans-protective activity against Dpl.

may produce no toxic signal (Fig. 2B). The N-terminal region, together with the central region, may compete with Dpl or the truncated PrPs for the transmembrane molecule, resulting in reduction of the neurotoxicity of Dpl or the truncated PrPs in trans (Fig. 2C). In contrast, PrP molecules which lack the N-terminal region, such as PrPΔ23-88, have no potential to protect against Dpl or the truncated PrPs in trans (Fig. 2D). According to this model, the neurotoxicity of Dpl or the toxic PrPs is explained by interaction between Dpl or the Dpl-homologous C-terminal region of PrP and the putative transmembrane molecule (Fig. 2A). The cis- and trans-neuroprotective activity may be mediated by disturbing the interaction in cis or trans via the central or N-terminal regions, respectively (Fig. 2B–D). The neurotoxic PrP peptide, PrP106-126, and neurotoxic monoclonal anti-PrP antibodies, IgG D13 and P, which recognize an epitope (residues 95–105) very adjacent to the central region, may impair the cis-activity of the central region and promote the neurotoxic binding between the C-terminal region of PrP^C and the transmembrane molecule, inducing neuronal cell death.[30,31] However, this model can be verified only when the putative transmembrane molecule is identified.

Implication for Prion Diseases

Many lines of evidence indicate that conformational conversion of PrP^C into the abnormally folded amyloidogenic isoform, PrP^{Sc}, plays a pivotal role in the pathogenesis of transmissible spongiform encephalopathies or prion diseases, including Creutzfeldt-Jakob disease in humans and bovine spongiform encephalopathy in cattle.[32] However, the molecular mechanism by which neurons undergo degenerative death remains unknown. PrP^{Sc} differs from PrP^C in tertiary structure.[33] PrP^C is rich in α-helix content while PrP^{Sc} have a markedly increased content of β-sheet.[33] Thus, due to the structural changes, the central region of PrP^{Sc} may lose its cis-activity and PrP^{Sc} therefore might interact with the putative transmembrane molecule, causing neuronal degeneration. Alternatively, association between PrP^C and PrP^{Sc} during the structural conversion might impair the *cis*-activity of the associating PrP^C, subsequently inducing neuronal cell death. N-terminally truncated forms of protease-resistant PrP have been reported to accumulate in the brains of patients affected with prion diseases and in persistently infected cultured cells.[34,35] It may be also conceivable that these N-terminally truncated PrP fragments posses a neurotoxic potential equivalent to that of Dpl and Constructs 1 and 2 due to deletion of the cis-element. Thus, elucidation of a molecular mechanism of the antagonistic interaction between Dpl and PrP^C could be useful for understanding of the molecular pathogenesis of prion diseases.

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ORIGINAL ARTICLE

Cellular prion protein prevents brain damage after encephalomyocarditis virus infection in mice

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Abstract Cellular prion protein (PrP^C), a cell-surface glycoprotein normally associated with neurons, is also expressed in other cell types such as glia and lymphocytes. To further elucidate these roles of PrP^C, wild-type prion protein gene (*Prnp*$^{+/+}$) mice and *Prnp*-deficient (*Prnp*$^{-/-}$) mice were infected with encephalomyocarditis virus B variant (EMCV-B) via an intracranial route. EMCV-B causes encephalitis and apoptotic cell death in vivo. Histopathological studies revealed that *Prnp*$^{+/+}$ mice infected with 600 pfu of EMCV-B showed more severe infiltration of inflammatory cells, accompanied by higher activation of microglia cells around the hippocampus, than *Prnp*$^{-/-}$ mice; viz., no differences in the brain virus titer between these two lines of mice. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP, nick end-labeling (TUNEL) staining of the brain specimens revealed that the CA1 hippocampal pyramidal cells showed a larger number of apoptotic neurons in *Prnp*$^{-/-}$ than *Prnp*$^{+/+}$ mice. Based on all these findings, PrP^C may play certain roles in the induction of inflammation and inhibition of apoptosis in vivo.

Y. Nasu-Nishimura, Y. Taniuchi contributed equally to the study.

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Introduction

Prion diseases, such as bovine spongiform encephalopathy (BSE), scrapie and Creutzfeldt–Jakob disease (CJD), are a category of transmissible diseases with common pathological findings in the central nervous system (CNS), including neuronal degeneration, vacuolation and gliosis [23]. In addition, these diseases are fatal and manifest as an infectious, sporadic or genetic disease affecting both animals and humans [26, 27].

The scrapie isoform of prion protein (PrP^{Sc}) is an altered isoform of a normal cellular glycoprotein or the cellular isoform of the prion protein (PrP^C) [26]. PrP^{Sc} shows greater protease resistance and often accumulates in affected individuals in the form of extracellular plaques. PrP^C is a protein mostly expressed by neurons, and there is recent convincing evidence for its expression by other cell types including T-lymphocytes, splenocytes, astrocytes and glia cells [4, 7, 16, 20, 28]. Neuronal degeneration and neuronal cell death through apoptosis and gliosis are the

major pathological changes in spongiform encephalopathies that occur early in the course of these diseases. However, it remains unknown to date whether these pathological changes were induced by PrP^{Sc}, neurotoxocity, acute depletion of PrP^{C}, or some other mechanism. Moreover, the normal function of PrP^{C} remains unclear [28].

Recently, five independent groups have established six lines of mice devoid of the prion protein (PrP) gene (*Prnp*). These *Prnp*-deficient ($Prnp^{-/-}$) mice are designated as ZrchI, ZrchII, Npu, Ngsk, Rcm0 and Rikn using different gene-targeting strategies [5, 15, 17, 19, 25, 34]. The entire open-reading frame (ORF) of *Prnp* is replaced by selectable markers in Ngsk, Rcm0, ZrchII and Rikn $Prnp^{-/-}$ mice, although a part of ORF may remain intact in ZrchI and Npu $Prnp^{-/-}$ mice [28]. All of these mice exhibit normal early development with complete protection against scrapie infection, indicating that PrP^{C}, a dispensable protein in embryonic development, is essential for inducing prion diseases [1]. Ngsk $Prnp^{-/-}$ mice exhibit late-onset cerebellar ataxia due to an extensive loss of Purkinje cells in the cerebellum [25]. The fact that introduction of a PrP^{C} transgene prevents Purkinje cell degeneration and demyelination implies that PrP^{C} is directly involved in both the long-term survival of Purkinje neurons and the myelinating capacity of oligodendrocytes and Schwann cells [21].

A synthetic PrP peptide corresponding to amino acid residues 106–126 (PrP106–126) is toxic to PrP^{C}-expressing neurons and neuronal cells and induces apoptotic neuronal cell death in cultures [3]. In glia cell cultures, PrP106–126 induces astroglial proliferation [2], which is dependent on PrP^{C} expression [10]. In addition, glial cells have been implicated as an effector of inflammation in the nervous system. According to Kuwahara et al. [15], PrP-deficient neuronal precursor cells, which are supposed to die in serum-free culture via apoptosis, are spared by the induction of a *Prnp* transgene in these cells [15]. These results, at least, suggest that PrP^{C} may have important roles in the inhibitory process of apoptosis and inflammatory responses.

To further understand the functions of PrP^{C}, in vivo investigations of the PrP^{C}-inflammation and the PrP^{C}-apoptosis relationships are warranted. Primary embryonic fibroblasts [30, 32] and various other cell types [18, 22] undergo apoptotic cell death when infected by the encephalomyocarditis virus B variant (EMCV-B) [30, 32], which produces encephalitis and myocarditis in mice [31]. In the present study, the inflammatory responses to viral infection in wild-type ($Prnp^{+/+}$) and $Prnp^{-/-}$ mouse brains and the in vivo relationships between PrP^{C} and apoptosis were investigated using EMCV-B as an infectious agent.

Materials and methods

Animals

Rikn, ZrchI $Prnp^{-/-}$ and $Prnp^{+/+}$ mice were used in the study. $Prnp^{+/+}$ and $Prnp^{-/-}$ mice (F1 offspring of $Prnp^{+/-}$ parents) were infected with EMCV-B. Fifteen-week-old $Prnp^{+/+}$ and $Prnp^{-/-}$ mice were given an intracranial injection of EMCV-B (600 pfu/mouse) before sacrifice on post-infection days (DPI) 4 and 7. Non-infected 15-week-old $Prnp^{+/+}$ and $Prnp^{-/-}$ mice (controls) were treated in a similar fashion with PBS and sacrificed accordingly.

Cell line and virus titration

Dulbecco's modified Eagle medium (DMEM; Gibco, New York, NY, USA) supplemented with 10% heat-inactivated (Hi) fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA) was used for L929 cell cultures. MEM (Nissui, Tokyo, Japan) supplemented with 1% Hi-FBS and 60 μg/ml of kanamycin and 4% methylcellulose was used to overlay cell monolayers for virus plaque assays [18]. Dulbecco's phosphate-buffered saline (PBS; Nissui, Tokyo, Japan) was used for tissue homogenates, dilution of virus and inoculation of control mice. Methylene blue solution containing 1% methylene blue and 10% formaldehyde was used for staining cells.

The left hemisphere of each brain was removed after sacrifice by cervical dislocation on the designated DPI, and weighed accordingly. The respective samples of isolated tissues were homogenized with equal volumes of PBS and stored at −80°C until titration usin the plaque assay. Virus titrations were performed using serially decimal dilutions of the preparation. A 25-μl sample of the virus preparation was introduced into the monolayers of L929 in 6-well plates (diameter: 35 mm; Iwaki, Tokyo).

Clinical symptoms

Animals were each placed in a transparent plastic cage ($19 \times 24.5 \times 13$ cm^3) with 2-cm-thick paper bedding. The cage was covered by a wire-meshed lid. Mobility levels (duration: 10 min) and paralysis levels of the hindlimb were monitored on a daily basis.

Histopathology

The right hemispheres were isolated from the respective mice on DPI 7 and fixed in 10% neutral buffered formalin before being paraffin-embedded. Sections (thickness: 8 μm) were cut, mounted onto microscope slides, and stained with hematoxylin and eosin (HE). In order to establish a difference in the relative intensity of rod-nuclear

cells (activated microglia) between $Prnp^{+/+}$ and $Prnp^{-/-}$ brain sections, HE-stained sections from each mouse were analyzed using an Olympus Standard microscope. Cell counts were expressed as the mean ± SD of 20 microscopic fields at 400× magnification. The pyramidal cell layer of hippocampus was selected for determining the infiltration intensity, which was not known to the observer when measuring the respective brain sections.

In situ nick end-labeling of the hemisphere sections

Based on the method of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP, nick end-labeling (TUNEL) was performed using an ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Intergen, Purchase, NY, USA) [6]. Deparaffinized sections were treated with proteinase K for 15 min at room temperature and incubated with 3% hydrogen peroxide for 5 min to terminate the endogenous peroxidase activity before further treatment with TdT solution for 1 h at 37°C. The sections were next treated with anti-digoxigenin peroxidise-conjugated antibody for 30 min at room temperature before nick end-labeling for visualization; i.e., immersing the treated sections in 3′,3′-diaminobenzine solution and counterstaining with hematoxylin or methylene blue.

Statistical analysis

Differences in the brain virus titers or infiltrated cells in the hippocampus between $Prnp^{+/+}$ and $Prnp^{-/-}$ mice were statistically determined by the unpaired Student's *t* test.

Results and discussion

$Prnp^{+/+}$ and $Prnp^{-/-}$ mice (F1 offspring of $Prnp^{+/-}$ parents) of 15 weeks of age were infected with EMCV-B. Differences in the capacity of EMCV-B to replicate in brains between the $Prnp^{+/+}$ and $Prnp^{-/-}$ mice were compared by measuring the virus titers of the left hemisphere with the plaque assay at the indicated DPI. The virus replicated equally well in EMCV-B-infected $Prnp^{+/+}$ and ZrchI and Rikn $Prnp^{-/-}$ mice on DPI 4 and 7; viz., $Prnp^{+/+}$ mice indicated titers of 10^5–10^7 and 10^2–10^6 pfu per (left) hemisphere on DPI 4 and 7, respectively, while titer differences between ZrchI and Rikn $Prnp^{-/-}$ mice were insignificant when compared with $Prnp^{+/+}$ mice on DPI 4 and 7 (Fig. 1a).

Based on histopathological investigations of the hemispheres of EMCV-B-infected mice on DPI 7, severe mononuclear cell infiltrations around the capillaries with slight hypertrophy of endothelial cells in the meninges and cerebral cortex were observed in $Prnp^{+/+}$ mouse brains (Fig. 2). The severity of mononuclear round-cell infiltration in $Prnp^{+/+}$ mice was proportional to the virus titer of infected hemispheres. In addition to severe mononuclear cell infiltrations around the capillaries in the hemisphere of $Prnp^{+/+}$ mice, proliferations of rod-shaped cells around the damaged areas (probably due to activation of microglia cells) were frequently observed in inflammatory sites (Fig.2). However, mild mononuclear round-cell infiltrations (even with virus titers of 10^6 pfu/hemisphere) were noted in the hemispheres of ZrchI $Prnp^{-/-}$ and Rikn $Prnp^{-/-}$ mice; viz., gliosis-like changes were not observed in the hemispheres of ZrchI $Prnp^{-/-}$ and Rikn $Prnp^{-/-}$ mice (Fig. 3b, c). Furthermore, the neurons in hemispheres of ZrchI $Prnp^{-/-}$ and Rikn $Prnp^{-/-}$ mice displayed karyorrhexis with shrunk cellular bodies.

To determine whether PrP-deficient mice were less reactive to the inflammatory response, brain sections were tested for microglia infiltration activity. Histopathological findings indicated signs of a suppressed microglial response in ZrchI $Prnp^{-/-}$ mice. Infiltrated rod-nuclear cells were counted in the EMC virus-infected brain. Limited perivascular cells were observed in $Prnp^{-/-}$ mice, while extensively infiltrated cells were observed in the hippocampal area of $Prnp^{+/+}$ mice (Table 1).

$Prnp^{+/+}$ mice did not show any clinical alteration until DPI 21. In a tendency similar to $Prnp^{+/+}$ mice, Rikn $Prnp^{-/-}$ and ZrchI $Prnp^{-/-}$ mice did not show any paralysis until DPI 21.

To study the PrP^C-apoptosis relationship, apoptotic cell death induced by EMCV-B infection in brains of 15-week-old mice were investigated by TUNEL. The pyramidal cells

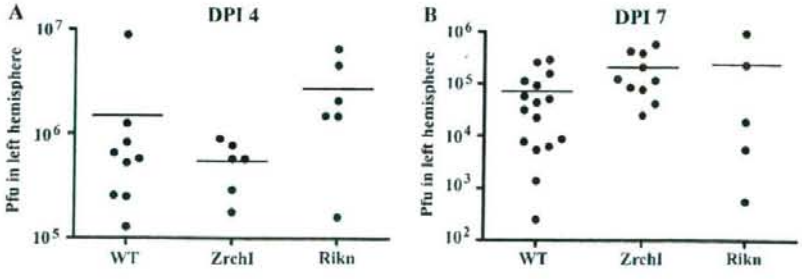

Fig. 1 Virus titers in the brain hemispheres of mice previously infected with encephalomyocarditis virus-B variant (*EMCV-B*). Virus titers in the brain hemispheres of 15-week-old mice infected with 600 plaque-forming units (*pfu*) per mouse of EMCV-B on post-infection days (*DPI*) 4 (**a**) and 7 (**b**)

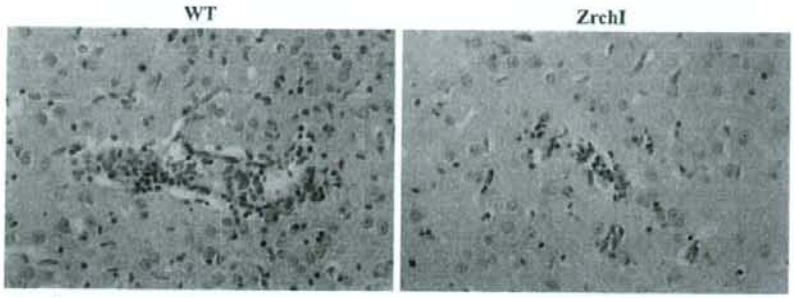

Fig. 2 Brain sections of 15-week-old EMCV-B-infected $Prnp^{+/+}$ (WT) and ZrchI $Prnp^{-/-}$ mice. Brain sections of $Prnp^{+/+}$ (WT) and ZrchI $Prnp^{-/-}$ mice (previously infected with 600 pfu of EMCV-B per mouse) showed severe mononuclear cell infiltration around the capillaries and mild mononuclear round-cell infiltration, respectively. In WT mice, the brain section indicated proliferation of rod-shaped cells around the damaged area; activation of microglia-like cells in the inflammatory area was frequently observed. Sections were stained with hematoxylin and eosin. ×315

Fig. 3 Brain sections stained by the terminal deoxynucleotidyl transferase (*TdT*)-mediated dUTP, nick end-labeling (*TUNEL*) method. Brain sections were obtained from 15-week-old wild-type ($Prnp^{+/+}$) and ZrchI $Prnp^{-/-}$ mice previously infected with 600 pfu/mouse of EMCV-B on post-infection day (*DPI*) 7. The nuclei were counterstained with methylene blue. **a**, **d** The hippocampus and fimbria area of $Prnp^{+/+}$ (WT) mice, respectively. **b** The hippocampus from ZrchI $Prnp^{-/-}$ mice. **c**, **e** The hippocampus and fimbria area, respectively, of Rikn $Prnp^{-/-}$ mice. While CA1 pyramidal cells in the hippocampus of ZrchI $Prnp^{-/-}$ mice showed signs of apoptosis (**b**), those of WT mice (although degenerated by EMCV-B infection) were not TUNEL-positive (**a**). The fimbria cells of ZrchI $Prnp^{-/-}$ mice (**b**) showed a higher degree of apoptotic cell death than those of WT mice (**a**). A brain section from $Prnp^{+/+}$ mice shows severe mononuclear cell infiltration around capillaries (**a**, *arrow*). A brain section of $Prnp^{+/+}$ mice displays proliferation of rod-shaped cells around the damaged area and frequent activation of microglia-like cells in the inflammatory area (**a**, **d**, *arrow*). A brain section from Rikn $Prnp^{-/-}$ mice shows mild mononuclear round cell infiltration (**c**). Sections used in the TUNEL method were counterstained with hematoxylin and eosin. CA1 pyramidal cells in the hippocampus of Rikn $Prnp^{-/-}$ mice showed apoptosis (**c**), while those of WT mice did not indicate any TUNEL-positive cells (although degenerated by EMCV-B infection) (**a**). A TUNEL-positive nucleus of neuronal cell is indicated by a black arrow, while TUNEL-positive nuclei of infiltrated cells are indicated by red arrows. The cells of fimbria from Rikn $Prnp^{-/-}$ mice (**e**) showed a higher apoptotic cell death rate than WT mice (**d**). **a**, **c**–**e** show ×230, while **b** shows ×630

of hippocampus (CA1) showed severe apoptotic neuronal cell death in both ZrchI $Prnp^{-/-}$ and Rikn $Prnp^{-/-}$ mice (Fig. 3b, c). Although showing the degenerative alterations, pyramidal cells of the hippocampus (CA1) in $Prnp^{+/+}$ mice were not TUNEL-positive (Fig. 3a). It is noteworthy that limited cellular infiltration was observed around the

Table 1 The infiltrated rod-nuclear cell density in the CA1 region of hippocampus in various mouse lines

Mouse	Cells counted (mean ± SD)
Wild type	96 ± 14.67
Rikn $Prnp^{-/-}$	15 ± 4.08
Zrchl $Prnp^{-/-}$	28 ± 2.16

Cell counts were expressed as the mean ± SD of 20 microscopic fields of 3 different sections from 3 infected mice at 400×

The pyramidal cell layer of hippocampus was selected for determining the infiltration intensity, which was not known to the observer measuring the cell count of the respective sections. There was significant difference between wild-type and $Prnp^{-/-}$ mice ($P < 0.01$)

apoptotic nerve cells in hemispheres of ZrchI and Rikn $Prnp^{-/-}$ mice. Moreover, TUNEL-positive infiltrating cells were observed more frequently in ZrchI and Rikn $Prnp^{-/-}$ (Fig. 3b, e) than in $Prnp^{+/+}$ mice (Fig. 3d).

In this study, the roles of PrP^C in microglia infiltration and apoptosis were elucidated. Although there was no difference in virus titers in the brains of $Prnp^{+/+}$ and $Prnp^{-/-}$ mice, variations of the inflammation intensity in brains between age-matched $Prnp^{+/+}$ and $Prnp^{-/-}$ mice were noted. $Prnp^{+/+}$ mice were inclined to have more intense brain inflammation than $Prnp^{-/-}$ mice. Brown et al. [2, 3] have demonstrated that PrP 106–126 neurotoxic peptide is toxic to cerebral cells derived only from $Prnp^{+/+}$ (but not $Prnp^{-/-}$) mice, and PrP 106-126-induced microglia-mediated astrocyte proliferation depends on PrP^C expression in astrocytes. In brains of $Prnp^{+/+}$ mice, proliferations of rod-shaped cells were frequently located around the damaged areas (probably due to activation of microglia cells) that coincided well with the distribution of inflammatory sites. Microglia is a major cell type which produces nitric oxide (NO). NO plays a role in the active cell-protective mechanism. Keshet et al. [12] have revealed that adult (age >100 days) $Prnp^{-/-}$ mice harbor significantly reduced NO synthase (NOS) activity, while young (≦30 day old) $Prnp^{-/-}$ mice exhibit NOS activity similar to that of wild-type or $Prnp^{+/+}$ mice. With regard to the inflammatory responses in 15-week-old EMCV-B-infected mouse brains, mild-to-severe inflammatory changes were observed only in 15- but not in 6-week-old $Prnp^{+/+}$ mice (data not shown). Recent studies have demonstrated decreases in certain enzyme activities (superoxide dismutase and catalase) in the brain of $Prnp^{-/-}$ mice [14, 24, 29]. This enzyme-related deficiency could have induced an intracellular oxidative state in brain cells. Moreover, increased lipoperoxidation, a phenomenon that has been reported in other neurodegenerative diseases such as Alzheimer's disease and epilepsy [8, 9], was observed. In the absence of PrP^C, increased oxidation of lipids and proteins has been observed in the brain [35]. These results suggest that the physiological function of PrP^C is related to the cellular antioxidant defense system. Loss of the antioxidant defense system in $Prnp^{-/-}$ mice might have contributed to exacerbated lesions in the brain after EMCV infection.

In forebrain ischemia, apoptotic neuronal cell death of the pyramidal cells in the hippocampus (CA1) was indicated by TUNEL staining. Pyramidal cells in the CA1 region are susceptible to various chemical stimulations. In this study, TUNEL staining also revealed apoptotic neuronal cell death of the pyramidal cells in field CA1 of $Prnp^{-/-}$ mice. However, injured pyramidal cells of $Prnp^{+/+}$ mice did not display any cell death. Tanji [33] has demonstrated that the pyramidal cells in field CA1 of hippocampus express PrP^C mRNA in Wistar rats. These results suggest that the loss of PrP^C expression in CA1 reduces the viability of pyramidal cells in this brain region. In the present study, we therefore focused on the effects of viral (EMCV-B) infection in CA1 pyramidal cells of $Prnp^{-/-}$ and $Prnp^{+/+}$ mice to understand the crucial functions of PrP^C.

The extensive appearance of mononuclear round cells or rod-nuclear cells, which suggests the infiltration of microglia/macrophage, is an issue of interest as well. It has been shown that microglia provides protective effect against brain lesions [11]. Kitamura et al. [13] have performed histological and functional analyses to show the neuroprotective effect of exogenous microglia on neuronal injury; viz., injected microglia ameliorates ischemia-induced synaptic deficits, probably to release certain diffusible factors and eventually reduce brain-derived neurotrophic factor (BDNF) levels in CA1 neurons. Further studies of BDNF in $Prnp^{-/-}$ mice after virus infection are warranted.

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Conflict of interest statement No financial conflict and potential conflict of interest in the present study is hereby declared.

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Expert Opinion

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Recent developments in therapeutics for prion diseases

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Background: Conformational conversion of the normal prion protein PrP^{C} into its pathogenic form, PrP^{Sc}, is the central event in the pathogenesis of prion diseases, including Creutzfeldt–Jakob disease in humans and bovine spongiform encephalopathy in cattle. This constitutive conversion results in accumulation of PrP^{Sc} in the brain and many lines of evidence indicate that the accumulated PrP^{Sc} may be detrimental, causing degenerative neuronal cell death. *Objective*: Therapeutic compounds for prion diseases must reduce PrP^{Sc} in affected brains. Many compounds have been identified to reduce PrP^{Sc} levels in infected cells and some were partially effective in infected animals, prolonging incubation or survival times. Clinical trials have only recently started with a few of these compounds. They are systematically introduced and their performances and possible mechanisms summarised. *Methods*: Bibliographic research was carried out using the PubMed database. Patent literature was searched using the UK Intellectual Property Office website. *Results/conclusion*: No compounds reported so far have proven to be therapeutically effective against prion diseases, due to inadequate access to brains through the blood–brain barrier. Moreover, due to lack of diagnostic indicators for presymptomatic individuals, the compounds must be given to clinically advanced patients, reducing their effectiveness. Thus, it is also important to resolve these problems to develop therapeutically effective compounds for prion diseases.

Keywords: prion, prion disease, prion protein, therapeutics

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1. Introduction

Prion diseases or transmissible spongiform encephalopathies, including Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal familial insomnia (FFI) and kuru in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals, are fatal neurodegenerative disorders caused by prions [1,2]. These diseases are characterised by distinctive pathological changes in the CNS, such as spongiosis, neuronal cell death and accumulation of the abnormally folded, amyloidogenic isoform of prion protein, designated PrP^{Sc} [2]. Human prion diseases have different aetiologies. Most cases of the disease (85 – 90%) belong to a sporadic type of CJD with unknown aetiologies [2]. Approximately 10% of cases are inherited types, including familial CJD, GSS and FFI, all of which are linked to specific mutations of *Prn-p*, the gene for PrP [2]. The remaining cases, including those of iatrogenic CJD, variant CJD (vCJD) and kuru, are caused by infectious events [2]. Most cases of this type are iatrogenic CJDs [3-6]. Kuru is a disease spread by ritualistic cannibalism in Papua New Guinea [7]. vCJD is thought to be transmitted from BSE-infected cattle via contaminated food [8-10].

Only ~ 160 cases of vCJD have been reported in England and a much lesser number of cases in other countries to date, probably due to the inefficiency of

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oral transmission, the species barrier between cattle and humans and marked reduction of BSE cases [10]. However, different from classical CJD prions, it was experimentally demonstrated that vCJD prions enter blood circulation, causing prionaemia. This suggested a risk of human-to-human transmission of vCJD via blood transfusion or other medical treatments [11]. Unfortunately, three cases of blood transfusion-related vCJD were reported, with one of them being diagnosed as vCJD by autopsy. The others developed the disease several years after blood transfusion from donors who succumbed to vCJD [12,13]. The fact that vCJD can be secondarily transmitted to humans through blood transfusion has raised great concerns about an epidemic of vCJD among human populations. In North America, another animal prion disease, chronic wasting disease, is spreading within mule deer and elk, raising similar health concerns if the disease can be transmitted to humans, causing another type of vCJD. Therefore, development of therapeutic and/or prophylactic measures for prion diseases is urgently awaited.

A large number of antiprion compounds have been identified using cell culture and animal models [14] and several compounds were or are being clinically trialled [15-19]. However, none of them have been reported to be clinically effective in extending survival times, probably due to inadequate accessibility of the compounds to the brains through the blood–brain barrier (BBB). In addition, due to the lack of measures to diagnose presymptomatic individuals, the compounds were given to clinically advanced patients, which may have reduced their therapeutic effectiveness. These problems remain to be resolved as major issues for development of effective therapeutic measures for prion diseases. This article systematically reviews most of the so far reported antiprion compounds, and their performance in infected cells or animals and possible mechanisms of function are summarised. In addition, the reported screening techniques for antiprion compounds are introduced.

2. Prions and mechanisms for their propagation

According to the protein only hypothesis, prions are thought to mainly consist of PrP^{Sc}, which is produced by conformational conversion of the normal cellular isoform of PrP, PrP^{c}, a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein [1,20,21]. PrP^{c} is rich in α-helix content, is highly soluble and protease-sensitive. In contrast, PrP^{Sc} easily forms a β sheet-rich amyloid, less soluble and more resistant to proteases. Consistent with the hypothesis, it was shown that the β sheet-rich amyloid of N-terminally truncated recombinant mouse PrP was infectious, causing the disease in mice [22]. Moreover, Silveira *et al.* reported that brain fractions containing aggregates of 14 – 28 PrP^{Sc} molecules were more infectious than those containing aggregates of < 5 PrP^{Sc} molecules [23]. These indicate that a prion might be a multimeric aggregate of PrP^{Sc}.

It is postulated that PrP^{Sc} interacts with PrP^{c}, inducing conformational changes producing new PrP^{Sc} molecules or propagating prions (Figure 1) [1,20,21]. The 37/67 kDa laminin receptor (LRP/LR) was reported to bind to PrP^{c} directly and indirectly via endogenous glycosaminoglicans (GAGs) [24] and to the protease-resistant core of PrP^{Sc} [25]. Moreover, it was shown that recombinant LRP/PR, anti-LRP/LP antibodies and exogenously added GAGs reduced PrP^{Sc} levels in infected cells [26,27]. These results suggest that LRP/LP and GAGs are involved in the conversion by binding to PrP^{c} and/or PrP^{Sc}. However, it remains unknown whether LRL/LR is the sole PrP-interacting protein that mediates the conversion.

PrP^{c} is thought to be converted to PrP^{Sc} on an endocytic pathway to lysosomes as PrP^{Sc} is detected in endosomes and lysosomes [28]. PrP^{c} is preferentially localised on the cholesterol-rich membrane domains in rafts [29-32]. Disruption or modifications of rafts were shown to affect PrP^{Sc} levels in infected cells [30,33], suggesting that proper localisation of PrP^{c} at raft domains might be important for conversion. Moreover, it was shown that chemical substances that are able to affect protease activities or pH in lysosomes could influence the PrP^{Sc} content of infected cells [34], indicating that some aspects of lysosomal function might be necessary for conversion.

3. Experimental models for evaluation of antiprion compounds

Kocisko *et al.* first demonstrated that protease K-resistant PrP^{Sc}-like PrP could be produced from PrP^{c} in an *in vitro* cell-free system by incubating highly purified PrP^{Sc} and PrP^{c} under certain conditions [35]. Subsequently, Saborio and colleagues developed a protein misfolding cyclic amplification (PMCA) technique, in which PrP^{Sc}-like PrP can be amplified by repeating *in vitro* conversion with very tiny amounts of original PrP^{Sc} [36] and demonstrated that prion infectivity could be increased by PMCA [37]. These results indicate that *in vitro* cell-free conversion might mimic some aspects of the *in vivo* conversion and that this cell-free system might be useful to screen antiprion compounds by investigating whether compounds could block the conversion.

The more reliable model is a cell culture model using infected cells. Mouse neuroblastoma N2a cells and neuronal GT1 cells that are persistently infected with prions derived from humans or animals, producing PrP^{Sc} and supporting prion replication [38-40], are routinely used for screening of antiprion compounds by investigating whether compounds could reduce cellular PrP^{Sc} levels. However, compounds effective in cell culture models are not always effective in animal models or in human trials because there is a huge gap between cell culture and animal models in terms of the BBB. Antiprion compounds must cross the BBB and enter brains to execute their activities.

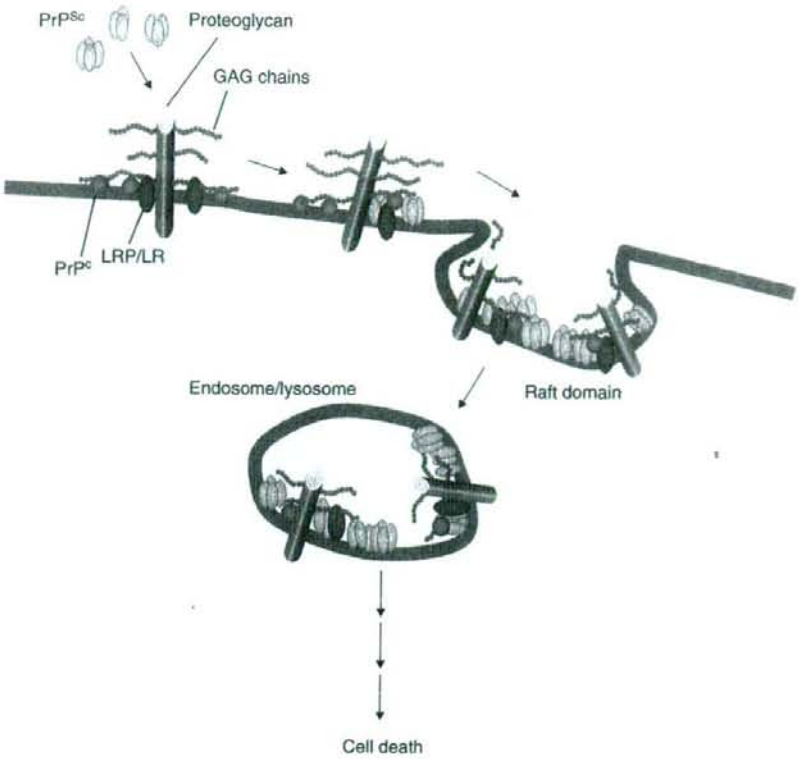


Figure 1. Mechanism for conversion of PrP^C into PrP^{Sc}. Invading aggregates of PrP^{Sc} interact with PrP^C directly or indirectly via binding to LRP/LR and/or GAGs, which are followed by conformational changes of the interacting PrP^C into PrP^{Sc}, resulting in accumulation of PrP^{Sc} in endosomes and lysosomes. The accumulated PrP^{Sc} finally leads to neuronal cell death.

The most reliable experimental model is an animal model. Animals such as mice and hamsters are infected by animal-adapted prions via peripheral or intracerebral routes and then candidate antiprion compounds are administrated peripherally or intraventicularly into the animals at various time points before or after infection. The therapeutic and/or prophylactic effects of the compounds are evaluated by investigating whether incubation or survival times could be prolonged or pathological changes, including accumulation of PrP^{Sc}, could be ameliorated in the treated animals. A few compounds, including pentosan sulfate (PPS) [19], quinacrine [17,41] and flupirtine [15] were or are being clinically tested.

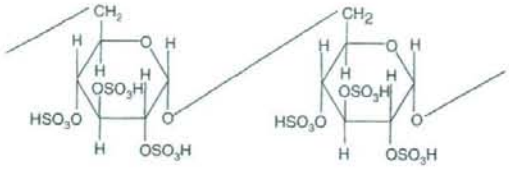
4. Polyanionic compounds

4.1 Polyanionic antiprion compounds and their possible mechanisms

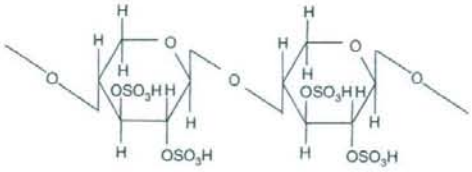
The first reported antiprion compound was the polyanionic antiviral agent, heteropolyanion-23 (HPA-23) [42]. This finding triggered further identification of other polyanionic antiprion compounds, including carrageenan [43], dextran sulfate (DS-500, **Figure 2A**) [43], PPS (**Figure 2B**) [43,44], GAGs [45-47] and heparan sulfate mimetics (HMs), such as artificially synthesised HM2602 and HM5004 [48,49]. These compounds decreased PrP^{Sc} levels in infected N2a or GT1 cells.

Nucleic acids are also polyanions. It was recently shown that single-stranded phosphorothioate oligonucleotides (PS-ONs, **Figure 2C**) [50-52] could reduce PrP^{Sc} levels and the infectivity of infected N2a cells [53,54]. This effect of PS-ONs was dependent on the phosphorothioate backbone and their length, but not sequence [53]. PS-ONs could preferentially bind to PrP^C rather than to PrP^{Sc} and the phosphorothioate backbone increases the hydrophobicity of ONs [53], suggesting that hydrophobic interaction of PS-ONs with PrP^C might be important for their antiprion activity. Moreover, the G-quartet structure containing DP7 RNA aptamer (**Figure 2D**) [55], which binds to residues 90 – 129 of human, hamster and mouse PrPs, was identified as an antiprion RNA, decreasing PrP^{Sc} levels in infected N2a cells [56]. Rhie *et al.* isolated 2′-fluoro-RNA aptamers that bind to

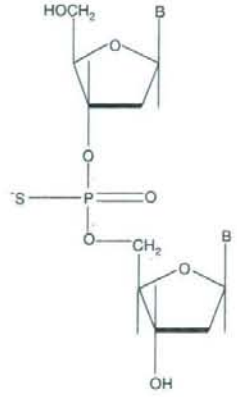
A: Dextran sulfate



B: Pentosan polysulfate



C: Phosphorothioate



D: RNA aptamers

DP7:
GUGCUAU**GG**AGUGGAGGAGUUGAA**GG**UGUC**GG**GGUU**GG**C

SAF-93:
CUACGAACUCAUGACACAAGGAUGCAAUCUAUCCCGCCAGCCCACCGU

Figure 2. Chemical structures of dextran sulfate (A), pentosan sulfate (B), phosphorothioate (C), and nucleotide sequences of two RNA aptamers, DP7 and SAF-93 (D). Four bold GG dinucleotides form a G quartet scaffold motif.

PrP^{Sc} rather than to PrP^{c} and showed that the SAF-93 aptamer (Figure 2D) inhibited PrP^{Sc}-like PrP formation in a cell-free system [57].

The mechanisms for antiprion activity of these polyanionic compounds remain unknown. Endogenous cellular GAGs are thought to play an important role for PrP^{Sc} formation or stabilisation by interacting with PrP^{c} and/or PrP^{Sc} [45,47]. As polyanionic compounds share structural similarities with GAGs in part, it is therefore considered that antiprion polyanionic compounds might compete with endogenous GAGs for the interaction with PrP^{c} and/or PrP^{Sc}, thereby preventing conversion. It is also suggested that the antiprion activity of polyanionic compounds might be due to disturbance of subcellular localisation of PrP^{c} and/or PrP^{Sc}, as polyanions are known to affect membrane trafficking [49,58]. Moreover, it was shown that PPS and HMs prevented binding between the protease K-resistant core of PrP^{Sc} and LRP/LP [25], indicating the possibility that the antiprion activity of polyanionic compounds may disturb binding between PrP^{Sc} and LRP/LP.

4.2 Antiprion polyanions in animal models

Polyanionic compounds poorly enter the brain because they cannot pass effectively through the BBB. Thus, it would be difficult to expect therapeutic effects from these compounds via peripheral administration once prions have entered the brain. Indeed, HPA-23, carrageenan, DS-500, PPS and HM2602 could prolong the lifespan of infected mice or hamsters only when administered via peripheral routes before infection or at time points before peripherally inoculated prions could reach the CNS [42,48,59-62].

Doh-Ura *et al.* intraventricularly infused PPS continuously for 4 weeks using an Alzet osmotic pump into the brains of 263K scrapie prion-infected Tg7 mice, which are transgenic for hamster PrP^{c}, and showed that treatment with PPS (460 µg/kg/day) from even 35 days postinoculation (middle stage of the infection) could significantly prolong incubation times of the animals by 36 days [63]. Moreover, PPS (230 µg/kg/day) administration from 42 days postinoculation (late stage of the infection) were effective, extending incubation times by 14 days [63]. PrP^{Sc} was less accumulated and pathological changes were mild in the brains of the treated mice, compared with those of non-treated mice [63]. These results raise the probability that PPS or possibly other antiprion polyanionic compounds could have therapeutic potential against prion diseases if they can be effectively delivered into the brain.

4.3 PPS in clinical trials

PPS has been safely used as an anticoagulant and anti-inflammatory agent by enteral, percutaneous or intravenous routes in humans. Twenty-six patients with vCJD (4 cases), sCJD (8), GSS (5), iatrogenic CJD (7), and familial CJD (2) were intraventricularly inoculated with various amounts of PPS (11, 32, 110, 120 or 220 µg/kg/day) [19]. According to the report, 13 of the patients are still alive [19]. However, due to lack of specific and objective criteria for evaluating response to treatment, the therapeutic effects of PPS could not be proven. Further prospective investigations of long-term intraventricular administration of PPS might be required for conclusive assessment of its therapeutic effects. Subdural effusion was observed in the patients as