Neurotoxic Prion Protein (PrP) Fragment 106-126 Requires the N-Terminal Half of the Hydrophobic Region of PrP in the PrP-Deficient Neuronal Cell Line

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Abstract: The cytotoxicity of aged PrP(106-126) was examined using an immortalized prion protein (PrP) gene-deficient neuronal cell line. The N-terminal half of the hydrophobic region (HR) but not the octapeptide repeat (OR) of PrP was required for aged PrP(106-126) neurotoxicity, suggesting that neurotoxic signals of aged PrP(106-126) are mediated by this region.

Keywords: Prion disease, prion protein (PrP), apoptosis, PrP-deficient cell line, PrP(106-126).

INTRODUCTION

The infectious agent, prion, causes transmissible spongiform encephalopathies (TSE) such as Creutzfeldt-Jakob disease and Gerstmann-Sträussler Scheinker syndrome (GSS) in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in cattle [1]. The major or entire component of prion is thought to be the prion protein (PrP). After prion infection, cellular prion protein (PrP^C) is converted into an abnormal isoform of prion protein (PrP^{Sc}), which has been proposed to be responsible for TSE [1]. Therefore, PrP^C is an essential factor for the induction of prion diseases. This finding is consistent with the fact that PrP gene (*Prnp*)-knockout mice are resistant to infectivity and toxicity induced by pathogenic inoculates [2]. Spectroscopic studies have demonstrated that PrP^C contains 42% α-helix with minimal (3%) β-sheet content, whereas PrP^{Sc} contains 43% β-sheet with reduced (30%) α-helix [3]. The increase of β-sheet content reduces water solubility (or induces hydrophobic tendency) and generates amyloid fibrils [3]. The amyloid decomposition of PrP^{Sc} is accompanied by neuronal cell death [4]. Interestingly, the amyloid protein in GSS patients is a PrP fragment with the N-terminus located within residues 58-90 [i.e. the octapeptide repeat (OR)] and the C-terminus corresponding to residues ~150 [5, 6].

A peptide fragment encompassing residues 106-126 of human PrP has been shown to be highly hydrophobic and fibrillogenic [7, 8], especially the peptide sequence containing the highly amyloidogenic palindrome AGAAAAGA from positions 113-120 [9]. Although controversial [10, 11], several reports have documented PrP(106-126) toxicity [7, 9, 12-15]. Interestingly, several reports have also demonstrated that PrP(106-126) neurotoxicity depends on the expression of endogeneous PrP [16] as is also the case for the neurotoxicity and transmissibility of PrP^{Sc} [17]. However, Fioriti et al. have recently shown that PrP(106-126) neurotoxicity is independent of the expression level of PrP and is not mediated by abnormal PrP species [18]. Therefore, the relevance of PrP(106-126) as a model for studying prion-induced cell effects and the role of PrP^C expression in PrP^{Sc} and PrP(106-126)-induced effects has been subjected to debate [9, 10, 18-22].

In a previous analysis of PrP, we have accounted for the anti-apoptotic and anti-oxidative functions of PrP using a PrP-deficient immortalized hippocampal cell line [23, 24]. Furthermore, deletion analysis of PrP^C has demonstrated that the OR and N-terminal half of the hydrophobic region (HR) is an essential region for PrP functions [25]. Thus, in this study we take advantage of typical characteristics of the PrP-deficient neuronal cell line to investigate participation of PrP^C in the PrP(106-126) effect on cell death. Furthermore, the availability of PrP-deficient cells expressing PrP-deletion mutants prompted us to perform additional studies to determine the specific regions of PrP contributing to PrP-dependent neurotoxicity of PrP(106-126). Therefore, to locate the PrP regions responsible for PrP(106-126)-induced neurotoxic activities, the effect of PrP(106-126) on cell death of $Prnp^{-/-}$ cells was compared to that of $Prnp^{-/-}$ cells expressing wild-type PrP or various deletion mutants under serum-free conditions. PrP(106-126) is toxic to PrP-expressing cells, although such is not the case for $Prnp^{-/-}$ cells or PrP-expressing $Prnp^{-/-}$ cells with deletion of the N-terminal half of HR. Based on our findings, the N-terminal half of HR may be involved in PrP-dependent neurotoxicity of PrP(106-126).

MATERIALS AND METHODS

Cell Cultures, Animals and Peptides

Murine *Prnp*-deficient neuronal cells HpL3-4 [23] and transfectants including HpL3-4-EM [25], HpL3-4-PrP [25],

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HpL3-4-Δ#1 [25] and HpL3-4-Δ#2 [25] cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fatal calf serum (FCS) at 37°C in a humidified 5% CO_2 incubator. Unless otherwise specified, serum deprivation was performed as previously described [26]. Aged or non-aged peptides [PrP(106-126): KTNMKHMAGAAAAGAVVGGLG [7] were added to the media at various concentrations. PrP(106-126) synthesized and purified by American Peptide Company (Sunnyvale, CA) was distributed by Toyoo Nakamura (Itoham Central Research Institute, Ibaraki, Japan). To form aged protein, PrP(106-126) (0.5 mg/ml) was left to aggregate at 37°C for at least 48 h [27].

Western Blot Assay

Western blot assay was performed as described previously [26]. Briefly, cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer supplemented with 2mM phenylmethylsulfonyl fluoride (PMSF). The RIPA was composed of 10 mM Tris-HCl (pH 7.4) containing 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS) and 0.15 M sodium chloride. The protein concentration was measured using the Bio-Rad DC assay, and SDS/polyacrylamide gel electrophoresis was conducted before electrical transfer onto polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Pharmacia Biotech, Piscataway, NJ). PrP was detected as described previously [26] with anti-PrP 6H4 (Prionics, Zürich, Switzerland) [28] and the horseradish peroxidase-conjugated secondary antibody. The probed proteins were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

SOD Activity Assay

Cells were sonicated in ice-old RIPA buffer supplemented with 2mM PMSF and centrifuged at 15,000 x g for 5 min at 4°C. Protein concentrations of the supernatants were measured by the DC protein assay (Bio-Rad). Each protein extract (20 μg) was assayed by the superoxide dismutase (SOD) assay kit-WST (Dojindo, Kumamoto, Japan). The SOD activity was compared with 1.0 U of bovine erythrocyte Cu/Zn-SOD (Sigma S2515) activity and estimated using the standard curve of SOD activity *versus* absorbance at 450 nm. The SOD activity was expressed as U/mg protein.

Cell Survival Assay

In cell survival assays, cells were seeded on 96-well plates at 5,000 cells/well. Two days later, cells were washed twice with serum-free DMEM followed by incubation in serum-free DMEM. Incubation with peptide PrP(106-126) was performed in serum-free DMEM treated with peptides. Viable cell counts were estimated by the Tetra Color One cell proliferation assay system (Seikagaku Kogyo Co., Tokyo, Japan). The sodium salt of 4-[3-(iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (a water-soluble tetrazolium (WST)) was utilized to count the viable cells, as the cell viability assay using WST produces formazan dye that correlates linearly with the number of viable cells over the range of 1,000 to 50,000 cells/well [29]. As such, WST-treated cells were further incubated for 4 h. The formazan levels were measured with a microplate reader (BioRad, Benchmark, Japan) at 450-nm absorbance. Cell number of dead cells was estimated by lactate dehydrogenase (LDH) assay. In this assay, the release of LDH into culture medium was measured by the LDH activity as an index of cell death with the LDH-cytotoxic test (Wako, Osaka, Japan).

RESULTS AND DISCUSSION

The neurotoxic peptide PrP(106-126), which corresponds to amino acid residues 106-126 of human PrP [7, 8], was used in this study. Although peptide PrP(106-126) has been reported to elicit toxic effects on neurons [7], cortical cells [30], endothelial cells [15], neuronal cells [31, 32] and induce glial proliferation [33, 34] and microglial activation [35, 36], these effects remain controversial [10, 11]. Therefore, we first examined if the neurotoxicity of PrP(106-126), a peptide corresponding to the N-terminal half of HR of PrP, depended on PrP expression, and the cell viability of PrP-deficient cells was compared to that of PrP-expressing cells in the presence of PrP(106-126) under serum deprivation. Aged peptide PrP(106-126) significantly decreased cell viability of HpL3-4-PrP cells in a dose-dependent manner, whereas the aged peptide did not enhance that of HpL3-4-EM cells under serum deprivation for 48 h (Fig. **1**). On the contrary, non-aged peptide PrP(106-126) did not influence the cell viability of either HpL3-4-EM or HpL3-4-PrP cells. These results were confirmed by quantification of cell death with LDH assay (Fig. **2**). We then investigated the mechanism of aged PrP(106-126) on cell-death reduction by examining the influence of aged PrP(106-126) on PrP-regulated SOD activity [24]. Aged peptide PrP(106-126) significantly inhibited SOD activity of HpL3-4-PrP cells but not HpL3-4-EM cells, while non-aged peptide PrP(106-126) did not influence SOD activity of HpL3-4-EM or HpL3-4-PrP cells (Fig. **3**). These results suggest that aged PrP(106-126) inhibited PrP^C functions to decrease cellular SOD activity and eventually exacerbated cell death.

Secondly, the effect of the peptide on cell death of HpL3-4 cells expressing deletion mutants was investigated to locate the PrP region essential for eliciting neurotoxicity of aged PrP(106-126). By performing several deletions in certain regions of mouse PrP to define the roles of the OR and HR related with anti-apoptotic and anti-oxidative functions of PrP [25], we re-confirmed the expression levels of generated clones of the deletion mutants in this study; viz., the expression levels were similar to clones of the full-length protein quantified by Western blotting using either anti-PrP 6H4 (Fig. **4**). In OR-deficient PrP(Δ53-94, Q52H)-expressing HpL3-4-Δ#1 cells, N-terminally truncated PrP was detected with 6H4, which recognizes residues 144-152 of mouse PrP [27]. In HR-deficient PrP(Δ95-132)-expressing HpL3-4-Δ#2 cells, truncated PrP was also detected with 6H4. The expression levels of deletion mutants were equivalent to those of HpL3-4 cells expressing wild-type PrP.

Finally, the effect of PrP(106-126) on HpL3-4 cells expressing two PrP deletion constructs was tested (Fig. **5**). Although cell viability of HpL3-4 cells expressing wild-type PrP or PrP without OR (amino acid residues 53-94; HpL3-4-Δ#1) were attenuated, aged peptide PrP(106-126) did not

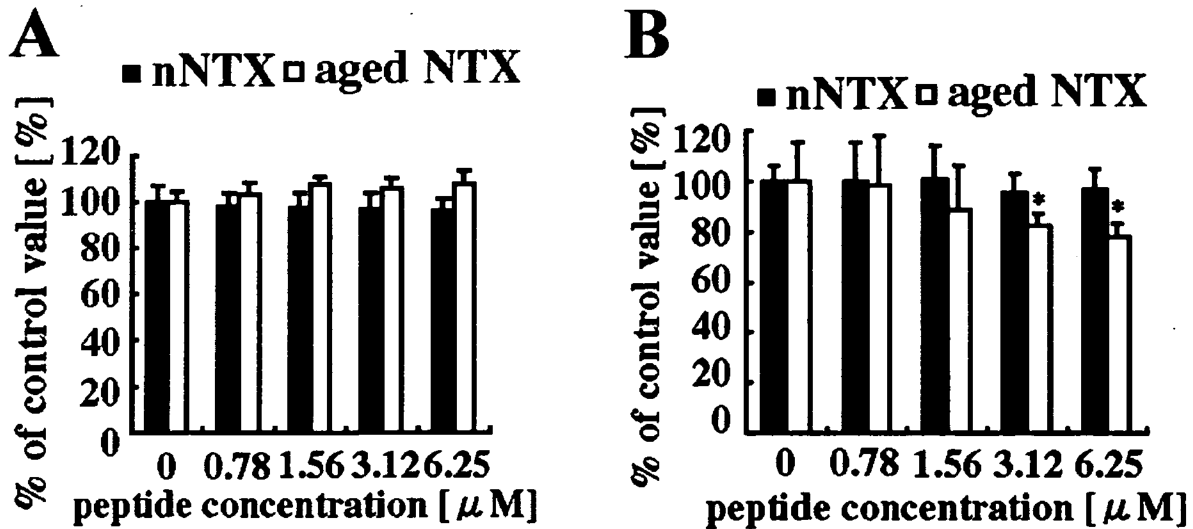

Figure 1. Toxicity of peptide PrP(106-126) in PrP-expressing cells.
The cell viability of 48-h serum-deprived HpL3-4-EM (*A*) and HpL3-4-PrP (*B*) cells in non-aged PrP(106-126) (nNTX) or aged peptide PrP(106-126) (aged NTX) at various concentrations were measured as described in Materials and methods. HpL3-4-EM cells have been reported to be more susceptible to serum deprivation than HpL3-4-PrP cells. The dose-dependent toxic effect of peptide PrP(106-126) was examined with the respective cell viabilities of HpL3-4-EM and HpL3-4-PrP cells cultured in serum-free media taken as 100%. Values are expressed as the mean ± SEM (N=4). Differences where $p < 0.05$ (*) *versus* Control (0 μM) were considered significant when verified by the non-repeated measures of ANOVA followed by the Bonferroni correction test.

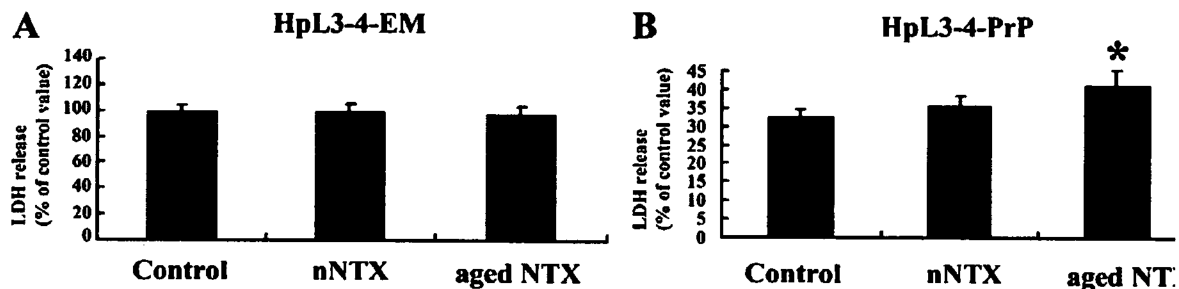

Figure 2. Enhancement of cell death by aged PrP(106-126) peptide in PrP-expressing cells.
HpL3-4-EM and HpL3-4-PrP cells were serum-deprived for 48 h. The cell death was quantitatively measured using the LDH assay. Values are expressed as the means ± SEM (N=4). The rate of cell death of serum-deprived HpL3-4-EM cells in the absence of peptide for 48 h (Control) was taken as 100%.

affect that of HpL3-4 cells expressing PrP without the N-terminal half of HR (amino acid residues 95-132; HpL3-4-Δ#2) or empty vector. In contrast, non-aged peptide PrP(106-126) did not effectively exacerbate cell death of all transfectants. These results were in accord with the results of LDH assay (Fig. 5). In the LDH assay, the cell death induced by aged PrP(106-126) in HpL3-4-PrP and HpL3-4-Δ#1 cells showed about 10%-increase compared with control, while that induced by aged PrP(106-126) in HpL3-4-Δ#2 cells did not show any enhancement of the cell death. These results suggest that N-terminal half of HR (amino acid residues 95-124) was indispensable for the elicitation of toxic activity of aged PrP(106-126).

In our present attempt to elucidate the molecular mechanism(s) of the cell death triggered by PrP(106-126), we were particularly interested in locating the PrP region essentially involved in said mechanism, using PrP-deficient neuronal cell line HpL3-4. The HpL3-4 cells have recently been established as a cell-line model for studying the functions of PrP^C. An exposure of HpL3-4-PrP cells to aged PrP(106-126) significantly exacerbated cell death in a dose-dependent manner. As PrP^C regulates cellular SOD activity, inhibition of the enzyme by aged peptide PrP(106-126) could exacerbate serum deprivation-induced cell death. In fact, PrP(106-126) significantly decreased SOD activity in the present investigation in HpL3-4-Prp cells. Cell viability assays of the essential regions of PrP^C for aged peptide PrP(106-126) indicated that the peptide required the N-terminal half of PrP (amino acids residues 95-124) to elicit the toxic activity. These results support the hypothesis of aged peptide PrP(106-126) binding to the N-terminal half of HR to elicit toxic activity, and that inhibition of the SOD activity plays a crucial role in cell death induced by serum deprivation in HpL3-4 cells.

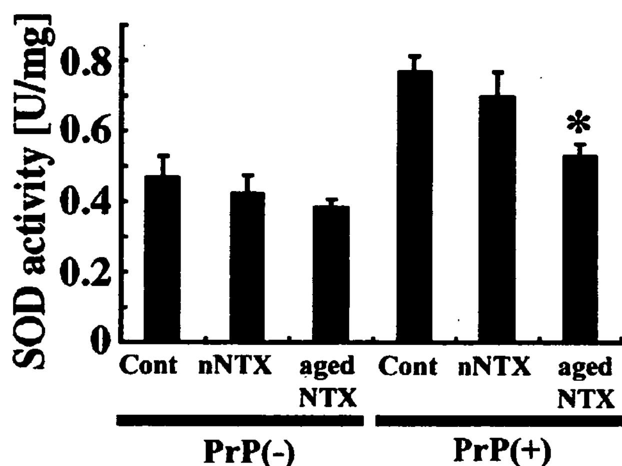

Figure 3. Inhibitory effect of peptide PrP(106-126) on SOD activity in PrP-expressing cells.
The SOD activities of 6-h serum-deprived HpL3-4-EM and HpL3-4-PrP cells in the absence (Control) or presence of non-aged PrP(106-126) (6.25μM) (nNTX) or aged peptide PrP(106-126) (6.25μM) (aged NTX) were measured as described in Materials and methods. Values are expressed as the mean ± SEM (N=4). Differences where $p < 0.05$ (*) *versus* Control (Cont) were considered significant when verified by the non-repeated measures ANOVA followed by the Bonferroni correction.

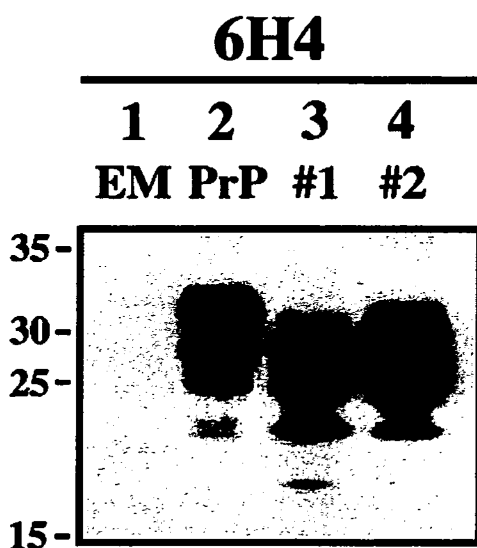

Figure 4. Expression of PrP and truncated PrP.
Western blot analysis with anti-PrP 6H4 (lanes 1-4) using an equal quantity of protein from HpL3-4 cells expressing wild-type PrP (PrP: HpL3-4-PrP), PrP(Δ53-94, Q52H) (#1: HpL3-4-Δ#1), PrP(Δ95-132) (#2: HpL3-4-Δ#2) or the empty vector per se (EM: HpL3-4-EM). Loadings were performed as follows: lane 1, HpL3-4-EM; lane 2, HpL3-4-PrP; lane 3, HpL3-4-Δ#1; lane 4, HpL3-4-Δ#2. It should be noted that 6H4 monoclonal anti-PrP recognizes residues 144-152 of mouse PrP [28]. Therefore, 6H4 would recognize wild-type PrP, PrP(Δ53-94, Q52H) and PrP(Δ95-132).

As the N-terminal half of PrP is an essential region of PrP for regulating SOD activity, which is mediated by PrP^C-stress-inducible protein-1 (STI1) binding, these results raise the possibility that binding of aged peptide PrP(106-126) to the N-terminal half of PrP inhibits PrP^C-STI1 signals to SOD activation, leading to exacerbation of cell death. However, these findings do not exclude the involvement of other mechanisms, such as activation of p38 MAP kinase [32], increased intracellular calcium contents [37] or induction of the synthesis and accumulation of neurotoxic transmembrane PrP [38], in the mechanism(s) of aged peptide PrP(106-126) toxicity. How aged PrP(106-126) treatment causes inhibition of SOD activity and why the aged peptide requires the N-terminal half of HR for toxic activity have yet to be defined. Based on our findings, it is important to note that concentration of aged PrP(106-126) needed to induce a toxic effect on HpL3-4 is lower than those required in other studies using primary culture neurons and neuronal cell lines [7, 37]. Accordingly, the toxicity potency/efficacy of aged PrP(106-126) may depend on the type of cells or neurons, and HpL3-4 cells may be more susceptible to aged peptide PrP(106-126) than other cell lines.

In summary, PrP^C indicated in cells an anti-apoptotic function [23-25, 39-41] mediated by upregulation of cellular SOD, while STI1 is involved in PrP^C-dependent SOD activation [40]. This enzymatic activation may have been abrogated by aged peptide PrP(106-126), probably as a result of inhibition of the interaction between PrP^C and STI1 [42]. Furthermore, the N-terminal half of HR, which harbors the STI1-binding site, is a region indispensable for the induction of toxic activity of aged peptide PrP(106-126), suggesting that the aged peptide binding to the N-terminal half of HR is essential for aged peptide PrP(106-126)-mediated toxic signals. The present data are consistent with the hypothesis that after aged peptide PrP(106-126) has bound to the N-terminal half of HR, PrP-dependent SOD activation by STI1 is inhibited. Expression of PrP^C is indispensable for prion infection in animals, because the conversion of PrP^C to PrP^{Sc} leads to PrP^C deficiency [43] to etiologically induce prion diseases. Moreover, both PrP^{Sc} and aged peptide PrP(106-126) bind to PrP^C to enhance cell sensitivity to oxidative stress [9, 44]. Therefore, experimental models analyzing the mechanism(s) of aged peptide PrP(106-126) toxicity may serve as a useful approach in understanding the roles of PrP^C expression in PrP^{Sc}- and PrP(106-126)-induced effects in prion diseases.

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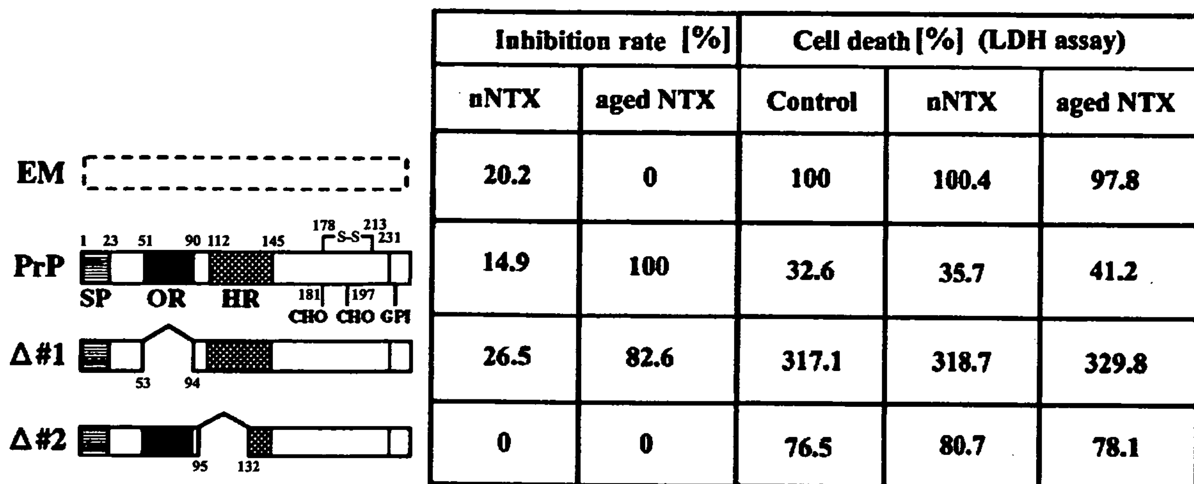

	Inhibition rate [%]		Cell death [%] (LDH assay)		
	nNTX	aged NTX	Control	nNTX	aged NTX
EM	20.2	0	100	100.4	97.8
PrP	14.9	100	32.6	35.7	41.2
Δ#1	26.5	82.6	317.1	318.7	329.8
Δ#2	0	0	76.5	80.7	78.1

Figure 5. Effect of deletion of OR and HR on toxicity of aged PrP(106-126) peptide.
Schematic presentations of PrP-deletion mutants of mouse PrP (PrP: wild-type PrP; Δ#1: PrP(Δ53-94, Q52H); Δ#2: PrP(Δ95-132)] are shown on the left. Schematic locations of the deletions compared with the wild-type protein are demarcated by a space within the bar next to the indicated protein. Numbers refer to the amino acid residues in the mouse PrP sequence. The disulphides (S-S), two Asn-linked glycosylation sites (CHO), signal peptide sequence (SP), octapeptide repeat region (OR), hydrophobic region (HR) and glycosylphosphatidylinositol anchor (GPI) are shown accordingly. Serum-deprived HpL3-4 cells expressing wild-type PrP (PrP: HpL3-4-PrP), PrP(Δ53-94, Q52H) (Δ#1: HpL3-4-Δ#1), PrP(Δ95-132) (Δ#2: HpL3-4-Δ#2) or the empty vector per se (EM: HpL3-4-EM) cells were incubated with 6.25μM of aged or non-aged peptide PrP(106-126). Inhibitory effects of the peptides on cell survival were examined 48 h later by the Tetra Color One cell proliferation assay. The rate of inhibition of HpL3-4-PrP cells by the aged peptide PrP(106-126) was taken as 100%. Furthermore, the cell death of these cells were also assessed by the LDH assay. In LDH assay, the rate of cell death of serum-deprived HpL3-4-EM cells in the absence of peptide for 48 h (Control) was taken as 100%.

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Novel Single Nucleotide Polymorphisms in the Specific Protein 1 Binding Site of the Bovine *PRNP* Promoter in Japanese Black Cattle: Impairment of Its Promoter Activity

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Key Words
Prion disease · Prion protein gene · Bovine spongiform encephalopathy · Promoter polymorphism

Abstract
Susceptibility to transmissible spongiform encephalopathy and different alleles of the prion protein gene *(PRNP)* of humans and sheep are associated. A tentative association between *PRNP* promoter polymorphisms and bovine spongiform encephalopathy (BSE) susceptibility has been reported in German cattle, whereas none of the known polymorphisms within the bovine *PRNP*-coding sequence affect BSE susceptibility. In the present study, novel single nucleotide polymorphisms located in the 5′-flanking region of bovine *PRNP* affecting its expression were demonstrated in Japanese Black cattle. We sequenced exon 1, and the approximately 200-bp 5′-flanking region of the *PRNP* translation initiation site containing the proximal promoter of *PRNP* was harvested. We identified 7 single nucleotide polymorphisms: –184A→G, –141T→C, –85T→G, –47C→A, –6C→T, +17C→T and +43C→T. Six segregated haplotypes in the population were cloned into luciferase-expressing plasmids, transfected into N2a cells, and their reporter activities were measured 48 h after transfection. Six haplotypes showed a decreased expression level including –6C→T in specific protein 1 binding site ($p < 0.05$) or –141T→C ($p < 0.01$) at 48 h compared with the wild-type haplotype. These results advocate that certain polymorphisms such as specific protein 1 binding site polymorphisms in the bovine *PRNP* promoter region in Japanese Black cattle could influence promoter activity, suggesting that breeding cattle with such substitutions may be a useful approach in reducing BSE risk.

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I.N. and G.X. contributed equally to this paper.

Introduction

Bovine spongiform encephalopathy (BSE) belongs to a category of transmissible spongiform encephalopathies (TSE) characterized by neuronal vacuolation and accumulation of an abnormal isoform of prion protein (PrP^{Sc}) in the central nervous system [1]. The PrP^{Sc}, which is converted from the cellular isoform of prion protein (PrP^{C}), is infectious. It is generally believed that BSE is caused by the ingestion of meat and bone meals from scrapie-infected sheep or BSE-infected cattle [2]. BSE poses a threat not only to cattle but also to humans, since the new variant Creutzfeldt-Jakob disease in humans is probably the

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result of an infection with BSE prions, which are contained in meat products from BSE-infected cattle [3, 4]. Consequently, many countries are developing policies aimed at eliminating BSE-affected animals from the food chain [5]. One such strategy is the breeding of BSE-resistant cattle lines. Certain TSE host genetic factors modulate the susceptibility to prion infection [4]. This phenomenon was initially discovered in sheep, where several mutations within the coding sequence of the prion protein gene *(PRNP)* are known to lead to increased or decreased scrapie susceptibility [6–10]. Similar genetic differences exist in humans, where *PRNP* polymorphisms have controversially [11] been reported to modify the development of Creutzfeldt-Jakob disease [12–16]. Thus, identifying genetic variations correlated with TSE resistance is an important step to eliminate TSE from the food chain.

A few *PRNP* polymorphisms have been characterized in cattle. *Bos taurus* in GenBank shows five nucleotide differences, including insertion/deletion polymorphisms within the coding sequence of the *PRNP*. The polymorphisms in cattle are not significantly correlated with BSE [8, 17–19]. Recently, a tentative association between *PRNP* promoter polymorphisms and BSE susceptibility has been found in German cattle [20]. Furthermore, polymorphisms found in the *PRNP* promoter region affect the promoter activity of Holstein Friesian cattle in Japan [21]. Very recently, one paper has reported polymorphisms of *PRNP* in Japanese Black cattle, which accounted for approximately 95% of cattle strain bred in Japan [19]. The 5′-noncoding region of the bovine *PRNP*, resembling genomic structures of the mouse, rat and sheep *PRNP*, consists of 3 exons. This 3-exon region actually contradicts the 2 exons found in the hamster and human *PRNP* [22–25]. For the bovine gene, a major promoter activity has been identified within the 5′-flanking region from –88 and –30, which contains only one putative specificity protein 1 (Sp1) binding site [21]. Sp1 is a sequence-specific transcription factor that binds the GC box and activates a wide range of viral and cellular genes [26]. Sp1 sites have been shown to be functional in many housekeeping gene promoters, where the transcription factor acts as a strong activator [27]. Therefore, the potential Sp1 binding site could be functional in the bovine *PRNP* in collaboration with the transcription factors. Potential Sp1 binding site polymorphisms may induce alterations in the allele-specific expression level and/or expression site [28]. Although the function of the potential promoter region of the bovine *PRNP* has been documented [21], the influence of Sp1 binding site polymorphisms on activity of the *PRNP* promoter region remains unclear.

In this study, normal *PRNP* promoter region polymorphisms, parts of the bovine *PRNP* promoter region, were initially analyzed in Japanese Black cattle breeds. Variations of the bovine *PRNP* promoter region in 45 Japanese Black cattle, including three potential Sp1 binding sites and exon 1, were elucidated. Activities of the bovine *PRNP* promoter region and the relevant promoter region were then analyzed. Certain polymorphisms in the bovine *PRNP* promoter region of Japanese Black cattle were found, and variation of –6C→T in Sp1 binding sites or –141T→C resulted in an approximately 20% decrease in luciferase activity compared with variants at other positions or the nonsubstituted *PRNP* promoter region.

Materials and Methods

DNA Samples

Fat tissues of 45 Japanese Black cows (JB1–JB45) were obtained from the Shirakawa Institute of Animal Genetics (Fukushima, Japan). Genomic DNA was isolated from the fat tissue by phenol/chloroform extraction. Briefly, bovine tissue samples were dissected and treated with 50 ml of DNA extraction buffer (150 mM NaCl, 10 mM Tris-HCl, 10 mM EDTA-2Na, 0.1% SDS) containing proteinase K (100 μg/ml). Mixtures were incubated at 55° for 1 h and at 37° for 16 h before treatment with an equal volume of neutral phenol. The mixture was agitated gently for 20 min before centrifugation at 2,000 *g* for 10 min at room temperature. Supernatants were further treated with an equal volume of PCI (phenol/chloroform/isoamylalcohol in the vol/vol ratio of 25:24:1) solution and subjected to gentle mixing for 20 min before centrifugation at 2,000 *g* for 10 min at room temperature. The isolated supernatant was gently mixed with two volumes of ethanol and incubated at –80° for 10 min before centrifugation (10,000 *g* for 10 min at room temperature). The supernatant was discarded and the pellet washed with 70% ethanol. Dried DNA pellets were dissolved appropriately with Tris-EDTA buffer.

DNA Amplification

A 546-bp fragment of the bovine *PRNP* promoter region, which included exon 1 and upstream-downstream of exon 1, was amplified by polymerase chain reaction (PCR) (fig. 1). Forward and reverse primer pairs were synthesized from reference nucleotide AJ298878 (GenBank) [28]. Two oligonucleotides, BPrP–463F (5′-AGAAGCTTGAGCAGGAACTGAGTAAATGACGG-3′) and BPrP+83R (5′-TGGGAGTTTAAAGGACTACGCGGC-3′), were used as primers to amplify the base pairs –463 to +83 of the gene (fig. 1) in PCR. The PCR was performed in a 50-μl reaction volume containing 150 ng DNA, 2.5 units Taq polymerase (Applied Biosystems, Norwalk, Conn., USA), 50 pmol of each primer, 2.5 mM dNTPs (Applied Biosystems), 2 mM $MgCl_2$, 2% DMSO and 10% reaction buffer provided by the manufacturer. The amplification was performed using an initial denaturation step at 94°

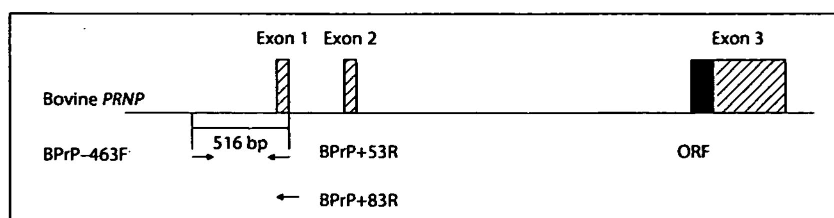

Fig. 1. Organization of the bovine *PRNP* gene. The hatched and black boxes denote the 3 exons of the *PRNP*. The protein coding region in exon 3 (black box) and exons 1 and 2 and the 5'- and 3'-noncoding regions in exon 3 (hatched boxes) are indicated accordingly. Two primers, BPrP-463F and BPrP+53R or BPrP -463-F and BPrP+83-R (arrows), were used to amplify the 516- or 546-bp fragments.

for 2 min, followed by 35 cycles of denaturation at 94° for 1 min before annealing at 53° for 2 min with extension done at 72° for 1 min. The final extension step was designated at 72° for 10 min. All PCR products were electrophoresed on 1% Sea Kem® GTG® agarose gels (FMC BioProducts, Rockland, Me., USA).

DNA Sequencing and Construction of Plasmids for Promoter Analysis

The 546-bp PCR product from 28 Japanese Black cattle samples was cloned into vector pT7Blue-T (Novagen, Madison, Wisc., USA) and sequenced on both strands using primers -21 M13 forward, M13 reverse or BPrP+83R and the ABI Prism® Big Dye terminator cycle-sequencing ready reaction kit (Applied Biosystems). Products of the sequencing reaction were run on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). The 516-bp fragment, including representative nucleotide substitutions, was cloned into vector pT7Blue-T after PCR amplification of the plasmids using BPrP-213-F (5'-AGCAATTTCAGGGAGTGATGA-GCC-3') and BPrP+53-R (5'-TCAAGCTTCTGCTTCTGCGAG-AGAGAAGACGC-3') before further subcloning into the luciferase reporter gene pGL3-basic (Promega, Madison, Wisc., USA) (fig. 1). Sequencing data analysis was performed with DNASIS-Mac version 3 software (Hitachi, Yokohama, Japan). Gene sequences utilized in the comparative analysis were derived from original published DNA sequences of GenBank (AJ298878, *Bos taurus*). In the present study, the *PRNP* sequences of the Japanese Black cattle were deposited in GenBank with the registration numbers AB250201, AB250202, AB250203, AB250204, AB250205, AB250206 and AB250207.

Cell Culture

Neuroblastoma cells (N2a) were cultured in Eagle's minimum essential medium with nonessential amino acids, sodium pyruvate, and supplemented with 10% fetal calf serum at 37° under 5% CO_2 for the luciferase assay.

Transient Transfection Luciferase Assay

In transient transfection assays, cells were seeded in 24-well plates at 2×10^4 cells/well 24 h before transfection. Cells reaching 60–80% confluency were transfected with 2 μg/well for each test construct, using vector pRL-SV (2 μg/well). Luciferase activity of cell lysates prepared at 48 h after transfection was measured as relative light units using a Fluoroscan Ascent FL (Labsystems, Franklin, Mass., USA). The promoter-free vector Luc (pGL3-basic) and the SV40 promoter containing vector Luc (pGL3-control; Promega) were transfected as negative and positive controls of promoter activity, respectively.

Results and Discussion

Deduced amino acid sequence of *PRNP* open reading frame from JB1–JB45 did not show any polymorphisms as compared with Holstein Friesian cattle (data not shown). In analyses of *PRNP* promoter region polymorphisms in Japanese Black cattle breeds, genomic DNA was isolated from fat tissues of 45 Japanese Black cattle (JB1–JB45). To detect polymorphisms existing in the Japanese Black cattle population in Japan, a 546-bp fragment of the bovine *PRNP* promoter region together with exon 1 was amplified using primers BPrP–463F and BPrP+83R for DNA sequencing (fig. 1). The PCR products were electrophoresed on 1% agarose gels, and the expected 546-bp bands were amplified from the cattle genomic DNAs. The PCR products cloned into vector pT7Blue-T were sequenced on both strands. The sequencing results were compared with bovine *PRNP* (AJ298878). From the 28 Japanese Black cattle, 7 different kinds of polymorphic sites were found in the bovine *PRNP* promoter region (table 1). Of these 7 polymorphisms, 5 have not been reported previously, and 2 previously described polymorphisms located at positions -184A→G and -85T→G were confirmed in the Holstein breed as well [28, 29]. Among the newly identified variants, 2 polymorphisms were located in the Sp1 binding site of the bovine *PRNP* promoter region (fig. 2). The C→T and C→A nucleotide substitutions were within 2 Sp1 binding sites: one was located at position -6 and the other at position -47 (fig. 2). Of the 28 Japanese Black cattle, 20 showed no nucleotide substitutions in the *PRNP* promoter region (fig. 2). The nucleotide substitutions were found at positions -141 and +17 of

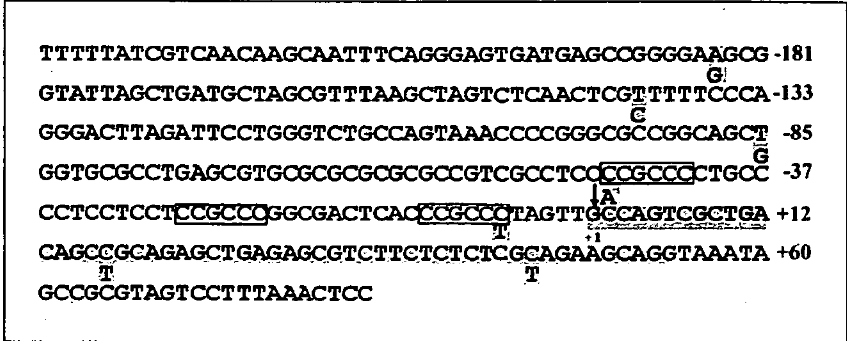

Fig. 2. Nucleotide sequences of the 5'-flanking region and exon 1. The transcription initiation site labeled as +1 is indicated by a vertical arrow. The 3 putative Spl binding sites (open boxes) and exon 1 (grey boxes) are indicated accordingly. The nucleotide substitutions (blue boxes) are indicated under the sequence AJ298878, and the sequence positions are given on the right.

Table 1. Sequence variants of the bovine prion protein gene *PRNP* promoter region in Japanese Black cattle

Position[a]	Variant	GenBank accession number	Allele number of the variant[b]
–184[c]	A→G	AJ298878	2
–141	T→C	this study	6
–85[d]	T→G	BN000291	2
–47	C→A	this study	1
–6	C→T	this study	1
+17	C→T	this study	5
+43	C→T	this study	1

[a] The position of the transcription initiation site is +1.
[b] Allele number in the 28 sequenced samples.
[c] This polymorphism has been documented in Hills et al. [28].
[d] This polymorphism has been documented in Sander et al. [29].

the *PRNP* promoter region in 4 Japanese Black cattle, but the nucleotide substitutions were also found at other positions, i.e. –184/–85, –184/–85/–6, –141/+43 and –141/–47/+17, in the 4 Japanese Black cattle. With reference to the allele frequencies of these polymorphisms (table 1), nucleotide substitutions at positions –6 and –47 located in the Spl binding sites were observed in each sample. Nucleotide substitutions at positions –184, –141, –85, +17 and +43 were observed in 2, 6, 2, 5 and 1 sample, respectively. These results suggest that certain polymorphisms in the bovine *PRNP* promoter region were located in Japanese Black cattle breeds.

To examine activities of the bovine *PRNP* promoter region, various reporter plasmids were constructed to analyze the promoter activity (fig. 3). DNA fragments, 516 bp (–463 to +53 bp), of the bovine *PRNP* promoter region including nucleotide substitutions at –184/–85, –184/–85/–6, –141, –141/+17 and –141/–47/+17 or no substitutions were cloned into a luciferase reporter vector pGL3-basic. These reporter plasmids were transfected with vector pRL-SV40 into neuroblastoma cells N2a. The inserted DNA in the pGL-nonplasmid did not contain nucleotide substitution in the *PRNP* promoter region. These fragments were cloned into vector pGL3-basic. The luciferase activities of cells transfected with several plasmids were compared with that of the pGL-nonplasmid. The activity of pGL3-control-transfected cells was taken as 100%. The plasmids pGL–184/–85 containing nucleotide substitutions at positions –184 and –85 showed 37.3% activity, which approximated to the luciferase activity comparable with the nonsubstituted sequences (36.3%) in N2a cells. However, the luciferase activity of the plasmid pGL–184/–85/–6 containing nucleotide substitutions at position –6 located in the Spl binding site showed significantly attenuated activity approximating 31.4% of the luciferase activity in N2a cells. Plasmids pGL–141 and pGL–141/+17, containing nucleotide substitutions at positions –141 as well as –141 and +17, manifested significantly suppressed activities approximating 27.4 and 26.9% of the luciferase activity in N2a cells. Furthermore, the luciferase activity of the plasmid pGL –141/–47/+17 showed a decrease of approximately 24.2% of that in N2a cells (fig. 3). The activities of the reporter plasmids pGL–141/+47/+17 and pGL–184/–85 were 10- and 16-fold more potent than the promoter-free reporter vector pGL3-basic in N2a cells, respectively. Taken together, these results suggest that a decrease in luciferase activity was induced by nucleotide substitution in Spl binding site or position –141 compared with the nonsubstituted *PRNP* promoter region in N2a cells.

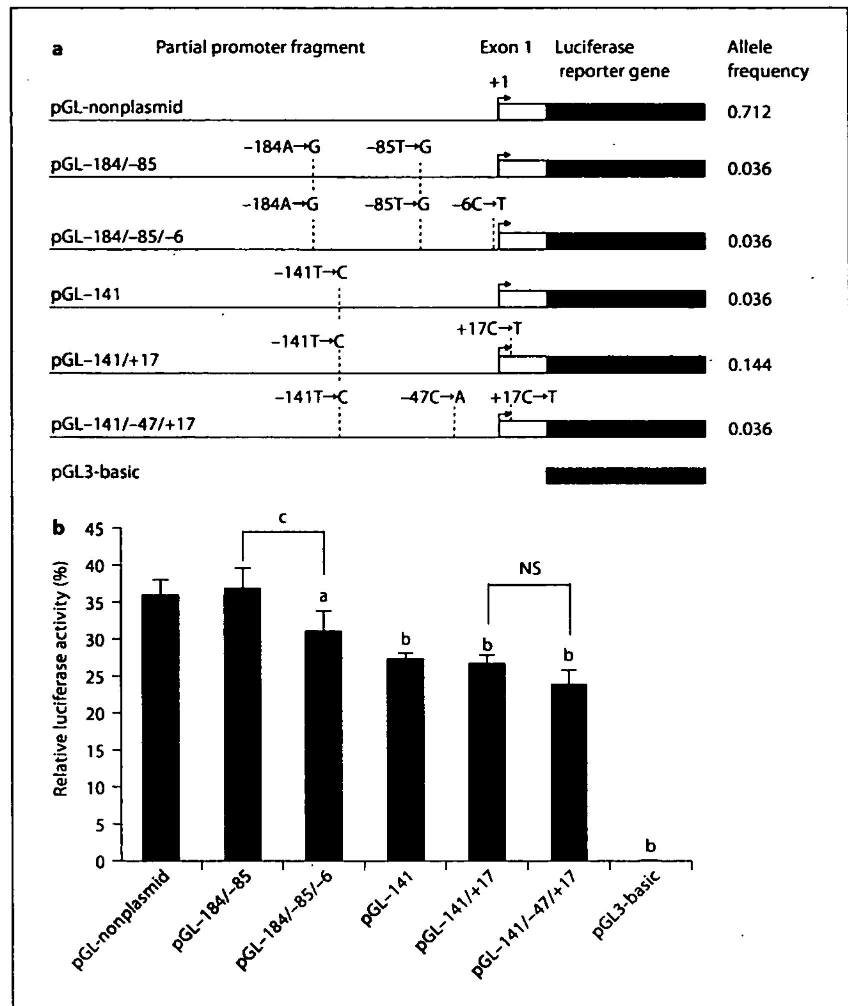

Fig. 3. Schematic representation of the reporter gene constructs. **a** Six reporter genes were constructed (pGL-nonplasmid, pGL-184/-85, pGL-184/-85/-6, pGL-141, pGL-141/+17, and pGL-141/+47/+17). pGL3-basic was the promoter-free vector control. Black box = luciferase gene; white box = exon 1, with arrows denoting the position of the reported transcriptional start site (+1) of the *PRNP* promoter region. Seven nucleotide substitutions are indicated above the reporter gene constructs. The allele frequency of 6 haplotypes is given on the right. **b** Luciferase activity was measured by luminometry of cell lysates prepared 48 h after transfection. Relative luciferase activities (mean ± SEM) for 3 replicate experiments were compared with the pGL3-control plasmid (100%). Differences were considered statistically significant (^{a} $p < 0.05$ and ^{b} $p < 0.01$) when compared with the pGL-nonplasmid using the nonrepeated measures ANOVA followed by the Bonferroni analysis. The difference between pGL-184/-85 and pGL-184/-85/-6 was statistically significant (^{c} $p < 0.05$), whereas the difference between pGL-141/+17 and pGL-141/-47/+17 was not significant (NS) using the nonrepeated measures ANOVA followed by the Student-Newman-Keul analysis.

In this study, results of variabilities of the bovine *PRNP* promoter region in Japanese Black cattle (consisting of 3 potential Spl binding sites) and the promoter activities in 28 Japanese Black cattle indicated 7 different kinds of polymorphic sites in the bovine *PRNP* promoter region. Two kinds of polymorphic sites in German cattle (including the Holstein breed) have been in the same region [20]. In comparison, variation within the bovine *PRNP* promoter region was apparently greater in Japanese Black cattle breeds than in Holstein breeds in the same region. All the identified variants harbored single nucleotide polymorphisms, without any detectable insertions/deletions. There are 3 potential Spl binding sites within the bovine *PRNP* promoter region (fig. 3) at positions between -11 and -6, -27 and -22, and -47 and -42 [21]. Among the newly identified variants, 2 kinds of polymorphic sites were located within the 2 Spl binding sites at positions between -47 and -6, respectively (fig. 2). Previously published work suggests that transcription in G+C-rich promoters usually initiates at multiple start sites between 40 and 100 nucleotides downstream of an Spl site [26]. The assumed Spl binding sequences are probably GGGGCGGGGC and TGGGCGGGGC [26]. Therefore, variations in the Spl binding site may influence the expression level. Promoter activities of the *PRNP* region from rat, bovine and mouse PrP^{C} genes have been reported previously. In the rat gene, deletions of box CCAAT (-66 to -41) and Spl binding sites resulted in a significant decrease in reporter gene activities, although these sites are opposite directions of consensus [24]. For the bovine

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gene, promoter activity has been identified within the 5'-flanking region from -88 and -30, which consisted of 3 putative Spl binding sites, which exist as opposite direction [21]. There was C→A substitution at nucleotide -47 and C→T substitution at nucleotide -6. Statistical comparison between pGL-141/+17 and pGL-141/-47/+17 suggested that C→A substitution in the Spl binding sites at nucleotide -47 did not significantly affect *PRNP* promoter activity (fig. 3b). On the contrary, statistical comparison between pGL-184/-85 and pGL-184/-85/-6 suggested that C→T substitution in the SP1 binding sites at nucleotide -6 resulted in a significant decrease in luciferase activity compared with those of the nonsubstituted *PRNP* promoter region (fig. 3b). The transcription factor neurogenin 1 was evaluated at base pair position -85 of the bovine *PRNP* promoter region by electrophoretic mobility shift assays [29]. For the G/T polymorphism at position -85, no conclusive differences in the binding property of the respectively different alleles have been observed [29]. These results suggest that variations in the Spl binding sites decreased the reporter gene activity, and Spl binding site polymorphisms could induce allele-specific expression levels. *PRNP* promoter polymorphisms modulate *PRNP* expression and may influence the BSE incubation period and BSE susceptibility [29]. Understanding the transcription of PrP^{C} expression is closely relevant to elucidation of the pathomechanisms leading to clinical symptoms in prion diseases.

In conclusion, there were certain polymorphisms in the bovine *PRNP* promoter region of Japanese Black cattle, and Spl binding site polymorphisms could influence the promoter activity. Interestingly, these polymorphisms may be involved in the lower levels of *PRNP* expression. Our studies suggest that breeding cattle with such lower expression levels of *PRNP* may be a useful approach in reducing BSE risk.

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REVIEW *Public Health*

Recent Developments in Prion Disease Research: Diagnostic Tools and *In Vitro* Cell Culture Models

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ABSTRACT. After prion infection, an abnormal isoform of prion protein (PrP^{Sc}) converts the cellular isoform of prion protein (PrP^{C}) into PrP^{Sc}. PrP^{C}-to-PrP^{Sc} conversion leads to PrP^{Sc} accumulation and PrP^{C} deficiency, contributing etiologically to induction of prion diseases. Presently, most of the diagnostic methods for prion diseases are dependent on PrP^{Sc} detection. Highly sensitive/accurate specific detection of PrP^{Sc} in many different samples is a prerequisite for attempts to develop reliable detection methods. Towards this goal, several methods have recently been developed to facilitate sensitive and precise detection of PrP^{Sc}, namely, protein misfolding cyclic amplification, conformation-dependent immunoassay, dissociation-enhanced lanthanide fluorescent immunoassay, capillary gel electrophoresis, fluorescence correlation spectroscopy, flow microbead immunoassay, etc. Additionally, functionally relevant prion-susceptible cell culture models that recognize the complexity of the mechanisms of prion infection have also been pursued, not only in relation to diagnosis, but also in relation to prion biology. Prion protein (PrP) gene-deficient neuronal cell lines that can clearly elucidate PrP^{C} functions would contribute to understanding of the prion infection mechanism. In this review, we describe the trend in recent development of diagnostic methods and cell culture models for prion diseases and their potential applications in prion biology.

KEY WORDS: BSE, cell line, diagnosis, prion disease, prion protein.

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Evidence of prion infections has been established by the accumulation of an abnormal isoform of prion protein (PrP^{Sc}) and/or infectivity after multiple passages [54, 85, 94, 131]. *In vitro* conversion of the cellular isoform of prion protein (PrP^{C}) to form PrP^{Sc}-like products has been demonstrated by incubating ^{35}S-labeled PrP^{C} with PrP^{Sc}. This produced a protease-resistant radioactive product with the mobility of protease-treated authentic PrP^{Sc} [55]. This *in vitro* conversion confirms the species- [90] and strain-specificities [7] observed *in vivo*. However, because the yield is less stoichiometric than for PrP^{Sc} [55], it has not been possible to determine whether or not an increase in infectivity occurred [55]. Recent progress in the development of diagnostic methods for PrP^{Sc} detection has dramatically facilitated many approaches in prion biology. The protein misfolding cyclic amplification (PMCA) method deserves special mention [96]. Hitherto, it has not been possible to renature completely denatured prion preparations to an infectious state [79, 81]; however, the novel PMCA approach enables *in vitro* amplification of infectious prions [16].

The susceptibility of cell lines, such as N2a, in an *in vitro* cell culture assay to prion infections has been assessed by exposing the cells to serial dilutions of prion-infected mouse brain homogenates, and the dilution rate yielding negative outcome in a blotting assay for PrP^{Sc} has been determined to be the infectivity level. Prion concentrations equivalent to those determined in an animal bioassay [9] have been quantitatively established using the same assay. The application spectrum of this prion cell assay for cell lineage can be extended from N2a to several other cell lines. Therefore, this greatly facilitates the mission of searching for prion-susceptible cell lines, and it is possible that this mission could be realized with the development of this assay and the recently innovated useful cell-culture models for prion infections. The present review introduces the recent advances in diagnostically useful methods and certain prion-susceptible cell lines that assist in understanding prion diseases.

PRP^{SC} DISTRIBUTION IN TISSUES

Before venturing into designing diagnostic methods and prion-susceptible cells, the tissue distribution of PrP^{Sc} in prion-infected animals should first be considered. Most of the diagnostic methods for prion infections depend on PrP^{Sc} detection, while susceptibility of cells to prions, at least in part, depends on cell-type. Since PrP^{Sc} tends to accumulate in the brains of prion-infected animals with neurons serving as the target, it is only natural that PrP^{Sc} accumulations are selectively located in the neurons of both the central nervous system (CNS) and peripheral nervous system (PNS) [10, 32, 34, 62, 137]. However, certain combinations of prion strain and host species show PrP^{Sc} accumulations in not only the CNS and PNS but also other tissues such as the tonsils, spleen, lymphnode, retina, proximal nerves, and gut-associ-

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ated lymphoid tissues [2, 12, 37, 39, 47, 89]. In the mouse, PrP^{Sc} accumulates in the lymphoreticular systems (such as the spleen) in an infection route-independent manner before PrP^{Sc} accumulation occurs in the brain, even if the mouse is intracerebrally inoculated [45]. PrP^{Sc} was detected in one-third of skeletal muscle and spleen samples [32] as well as the olfactory epithelium in sporadic Creutzfeldt-Jakob disease (sCJD) patients [142]. In a study of scrapie-infected sheep, prion infectivity was observed in 80% of the affected animals with PrP^{Sc} accumulation in the brain and PrP^{Sc} was detected in placentae and lymph nodes [84]. It should be noted that PrP^{C} is also highly expressed in the placentae of other species [123]. In fact, scrapie agents have been isolated from the placentae of sheep with natural scrapie [73]. Thus, the mother-offspring infection route may play an important role in natural transmission of scrapie.

PRP^{SC} IN BLOOD

PrP^{Sc} has been detected in blood in experimental mouse systems using the intracerebral (ic) and oral infection routes [17, 128]. PrP^{Sc} initially accumulates in the follicular dendritic cells (FDCs) of the germinal centers in lymphoid tissues [53, 69]; however, the biological event(s) that yields the presence of PrP^{Sc} in blood remains unclear. Scrapie has been transmitted by experimental blood transfusions in sheep [44]. Furthermore, detectable levels of infectivity in the spleen and tonsils of two variant CJD (vCJD) patients have been measured with a bioassay that has been employed using ic-inoculation and RIII mice [13]. Although this same bioassay failed to detect infectivity in the buffy coat and plasma [13], another study has demonstrated that mice infected with mouse-adapted vCJD or Gerstmann-Sträussler-Scheinker syndrome (GSS) harbor infectivity in the buffy coat and plasma [17]. Report of the possibility of blood borne transmission of vCJD in humans in the United Kingdom [61] has raised the need for a reliable prion detection method for clinical safety and may also contribute to development of a pre-mortem diagnosis method for prion diseases.

URINE PRP^{SC}

PrP^{Sc} has also been reported to exist in the urine of hamsters, cattle, and humans infected with prions [119]. However, urine PrP^{Sc} may have certain features different from those of brain PrP^{Sc}, as injections with the former do not cause prion disease [119]. Recently, several laboratories have reported that urine-derived PrP^{Sc} comprises immunoglobulins or bacterial outer membrane proteins [29, 35, 118]. On the other hand, others have reported that urinary proteins from scrapie-infected mice with lymphocytic nephritis induced scrapie upon inoculation into noninfected indicator mice [117]. Therefore, infectivity associated with the absence and presence of PrP^{Sc} in urine remains controversial. However, we do understand that certain proteins (urine PrP^{Sc}, immunoglobulins, or bacterial outer membrane proteins) may serve as a biomarker of prion infection if they are specifically detected in prion-infected animals.

CONVENTIONAL DIAGNOSTIC METHODS FOR PRION DISEASES

Most of the methods used for diagnosis of prion infections (Table 1) rely on the presence of PrP^{Sc} because the resistance of PrP^{Sc} to PK digestion is the specific feature distinguishing PrP^{Sc} from PrP^{C} [66, 67]. PrP^{C} is completely degraded, whereas the C-terminal of fragment PrP^{Sc} that remains after PK digestion can be detected by various method, such as Western blotting and enzyme-linked immunosorbent assay (ELISA). As PrP^{Sc} is abundantly accumulated in the brain, brain samples are appropriate for these detection methods, which require post-mortem analysis. Immunohistochemical (IHC) examination of brain sections is a relatively reliable method of prion diagnosis [3, 46] in which typical features of prion diseases, such as accumulation of prion protein (PrP) amyloid plaques, astrogliosis, and neuronal cell loss, are examined by light microscopy. Vacuolation is also used as an index of prion infection. However, many combinations of prion strain and host species portray PrP^{Sc} accumulation without vacuolation in brain sections after prion infection [48, 74]. Recently, positive diagnoses of PrP^{Sc} accumulation in prion diseases have been established using histopathological analysis of organs/tissues other than the brain. The tonsils may be used in humans, deer, and sheep [38, 39, 112, 113, 130, 137, 140], while the appendix has only been used for preclinical diagnosis of vCJD in humans [40, 41, 42, 47, 137]. Additionally, combination of the recently improved IHC analysis [using modified histoblot of paraffin-embedded tissue (PET) blot] with PK treatment or Western blotting has been introduced for detection of PrP^{Sc} in cryosections and paraffin-embedded sections, respectively [92, 114, 124].

Western blotting is a method used to detect PK-resistant PrP in extracts from the brain and other tissues. The mobility shift of bands for Western blotting provides information regarding the molecular weights of peptides. If the peptides are modified by aberrant glycosylation, different electrophoretic mobilities are observed. Therefore, electrophoretic mobility is influenced by host genotype and prion strain [75, 129]. In other words, the mobility index is often used for discriminating the CJD subtype [iatrogenic CJD (iCJD), sCJD, and vCJD], BSE, and scrapie strains [20, 36, 72, 77, 111, 136].

Although the use of animal bioassays has hitherto been considered the most sensitive approach to detect prions, the recently developed Western blot test (see below) is currently considered to match or surpass bioassay reliability/accuracy [137]. Nevertheless, it remains unclear whether PrP^{Sc} is completely identical in these cases or is the only entity of prions. Although PrP-expressing transgenic mice preinoculated with fibrils consisting of recombinant PrP have exhibited neuropathology similar to prion diseases [60], detailed comparison analysis between these animals and prion-

Table 1. Diagnostic methods for prion infections

Method	Index of method	Procedures	References
Western blot	PK-resistant PrP	Detect PK-resistant PrP on the membrane	[45]
ELISA	PK-resistant PrP	Detect PK-resistant PrP adsorbed on microtiter plates by anti-PrP antibody	[33]
Immunohistochemistry	PK-resistant PrP	Immunostain tissue sections	[64]
Bioassay	PK-resistant PrP, Incubation time or infectivity titer	Transmission to mice	[82]
Cell culture assay	PK-resistant PrP or infectivity titer	Transmission to cells	[54]
Histoblot	PK-resistant PrP	A cryosection is blotted onto membrane before PK-treatment and immunolabeled with anti-PrP antibody	[124]
Cell blot	PK-resistant PrP	Grow the cells on cover slip, directly transfer them to a membrane, and detect PK-resistant PrP using anti-PrP antibody	[9, 24]
Slot blot	PK-resistant PrP	Filter the cell lysate through a nitrocellulose membrane, and detect PK-resistant PrP using anti-PrP antibody.	[141]
PET blot	PK-resistant PrP	A PET section is collected on a membrane for PK-treatment and immunolabeled with anti-PrP antibody.	[92, 114]
PMCA	PK-resistant PrP	Amplfication of misfelding protein by cycles of incubation and sonication	[16]
CDI	PrP conformation	Specific antibody binding to denatured and native forms of PrP	[97]
DELFIA	Insoluble PrP	Measure the percentage of insoluble PrP extracted by two concentrations of guanidine hydrochloride.	[4]
Capillary gel electrophoresis	PK-resistant PrP	The competition between fluorescein-labeled synthetic PrP peptide and PrP present in samples is assayed by separation of free and antibody-peptide peaks using capillary electrophoresis.	[49, 109]
FCS	Aggregation of PrP	PrP aggregates are labeled by anti-PrP antibody tagged with fluorescent dyes, resulting in intensely fluorescent targets, which are measured by dual-color fluorescence intensity distribution analysis.	[8, 31]
Aptamer	PrP conformation	Use RNA aptamers that specifically recognize PrP^{C} and/or PrP^{Sc} conformation.	[138]
FT-IR spectroscopy	Alterations of spectral feature	Analyze the FT-IR spectra by multivariate analysis.	[110]
Flow microbead immunoassay	PK-resistant PrP	Detect PK-resistant PrP using a flow cytometer and anti-PrP antibody coupled with microbeads.	[69]
Surrogate marker	Change of expression level of 14–3–3 protein, erythroid-specific marker, or plasminogen	Two-demensional gel electrophoresis, differential display reverse-transcriptase PCR, or Western blotting of surrogate marker proteins for prion diseases	[26, 51, 68, 143]

PET: Paraffin-embedded tissue
PMCA: Protein misfolding cyclic amplification
CDI: Conformation-dependent immunoassay
DELFIA: Dissociation-enhanced lanthanide fluorescent immunoassay
FCS: Fluorescence correlation spectroscopy
FT-IR: Fourier transform infrared
ELISA: Enzyme-linked immunosorbent assay
PK: Proteinase K
PrP: Prion protein
PrP^{C}: Cellular isoform of PrP
PrP^{Sc}: Abnormal isoform of PrP

infected animals has yet to be attempted. Therefore, determination of infectivity with a reliable animal bioassay is the gold standard, although this assay may require a longer time period and many more animals. However, it should be noted that the volume of inoculum is critical for reducing the standard errors of the results in this animal bioassay. Characteristically, this assay can be complicated and may occasionally demand sophisticated techniques and effort [1]. Therefore, cell culture systems that specifically and reliably detect prions are urgently needed.

RECENTLY DEVELOPED DIAGNOSTIC TOOLS FOR PRION DISEASES

Recently, several modified Western blotting method have been developed for prion diagnosis. The cell blot [9, 24] and slot blot [141] assays have been further developed for detection of PrP^{Sc}. Cell blot is a method that involves transferring cells grown on a coverslip to a nitrocellulose membrane and then PK-resistant PrP is detected by Western blotting [9]. Slot blot is a method in which cell lysates are filtered through a nitrocellulose membrane, slot blot membrane, and a slot-blot device before PK-resistant PrP is detected by Western blotting [141]. The sensitivity of Western blotting can be also enhanced by the extraction method, in which PrP^{Sc} is precipitated with sodium phosphotungstic acid (PTA) [97, 137]. An efficient automated system with a detection limit of 10-pg could be established by combining PrP^{Sc} extraction using guanidine hydrochloride with the time-resolved dissociation-enhanced fluoroimmunoassay (DELFIA) test [4, 22]. However, the PrP^{Sc} concentration is no longer the parameter of this assay, but rather the percentage of insoluble PrP *versus* the total PrP content becomes the determinant index. Conformation-dependent immunoassay (CDI) is a method employed to detect conformational differences between PrP isoforms by measuring the relative antibody binding to denatured and native proteins [97]. CDI is used commercially for CDI testing at InPro Biotechnology, Inc. (U.S.A.). The flow microbead immunoassay (FMI) method, which uses anti-PrP antibody-coupled microbeads and a flow cytometer, was able to detect 7-pmol/7-nmol of recombinant PrP and PrP^{Sc} spiked in bovine meat and bone meal at concentration higher than 0.3% [69]. It should be noted that Soto *et al.* have recently developed novel PMCA technology that cyclically amplifies the misfolding and aggregation process *in vitro* [122]. It is conceptually analogous to DNA amplification by polymerase chain reaction (PCR). The PMCA method amplifies PrP^{Sc} and

prion infectivity titers using animal bioassays [16]. It is noteworthy that this approach is capable of prion detection in the blood of not only terminally diseased hamsters but also prion-infected presymptomatic hamsters [95].

Capillary gel electrophoresis is an approach that takes advantage of the competitive antibody-binding between a fluorescein-labeled synthetic PrP peptide and PrP present in tissue samples [49, 109]; free and antibody-peptide peaks are separated by capillary electrophoresis. Fluorescence correlation spectroscopy (FCS) is a method that detects individual fluorescence-labeled molecules in solutions [8, 31]. PrP^{Sc} can be labeled by the anti-PrP antibody or by conjugation with labeled recombinant PrP.

Fourier transform infrared spectroscopy is a diagnostic method that incorporates multivariate analysis of infrared spectra to discriminate prion-infected animals from noninfected animals [110]. This type of spectroscopy-based approach is promising for ante-mortem diagnosis, as certain ray emissions, especially visible and near-infrared radiations, are transmittable through the animal body, thus facilitating materialization of non-invasive analysis [98, 101].

DETECTION TOOLS FOR PRPSC AND SURROGATE MARKERS FOR PRION DISEASES

Potentially useful tools for detection of PrP^{Sc} have recently been developed. Although some antibodies and aptamers are able to distinguish PrP^{Sc} from PrP^{C}, these tools have not been extensively exploited for practical applications. Antibodies having a conformational epitope of PrP^{Sc} have been obtained by immunization with Tyr-Tyr-Arg peptide [76], and 15B3 and V5B2, which recognize PrP^{Sc}-specific epitopes, have recently been established [21, 56]. However, these antibodies have not been practically exploited, and their applications in experiments have been limited. Aptamer is an RNA or DNA molecule that specifically binds to a target-protein, and PrP^{C} and/or PrP^{Sc}-specific binding aptamers have been reported [91, 107, 138]. Interestingly, PrP^{Sc}-specific binding aptamer inhibits the accumulation of protease-resistant forms of PrP in prion-seeded *in vitro* conversion assay. Therefore, this type of aptamer subtype might not only serve as a diagnostic tool but might also facilitate therapeutic development and analytical use for investigation of prion diseases.

PRION PERSISTENTLY INFECTED AND SUSCEPTIBLE CELL LINES

Several prion-susceptible cell lines [9, 85] have been used for study of prion infection mechanisms (Table 2). The general cell culture procedures for prion infection have been described previously [6]. Brain homogenates of prion-infected animals were added to culture media for 1 –2 days before culture passage. Infectivity or PrP^{Sc} production per cell was assayed for percent infection of prions. Infectivity was measured after the brain samples of experimental animals were injected *via* ic inoculation. Harvested cells were lysed by repeating the freeze-thaw cycle before being injected into the brains of experimental animals. After performing behavioral observations for 1 year, the effective lethal dose (LD_{50}) was derived from the survival curve of the lysate-injected animals. PrP^{Sc} detection in the cells and animals was usually performed by Western blotting.

The persistently infected cell line thus established was divided into the following 2 categories based on post-infection status: established after *ex vivo* or *in vitro* infection. In the former case, it has been established that immortalized cell groups are generated in prion-infected animals. Thus, primary cultures from prion-infected brains can easily provide cells with continuous passage, i.e., ScHB and SMB produced from prion-infected animals [19]. Several cell lines represent the examples of the latter case. For example, nerve growth factor (NGF)-differentiated PC12 cells can be infected with prions [94], probably due to increased levels of PrP through NGF stimulation. Although derived from rat pheochromocytoma cells, PC12 cells are subject to infection by mouse-adapted scrapie. PC12 cell are the only cell line that defy the species barrier. Rabbit kidney-derived RK13 cells overexpressed with ovine PrP are susceptible to sheep scrapie infection [131]. Furthermore, mouse fibroblast NIH/3T3 and L929 cells are also susceptible to mouse-adapted scrapie infection [135]. In other words, non-neuronal cells can also be infected with prions under certain conditions. It is consistent that PrP^{Sc} is accumulated in not

Table 2. Cell culture models for prion infections

Cell lines	Origins	Prion strains	References
PC12	Rat pheochromocytoma	Scrapie (Chandler)	[94]
N2a	Mouse neuroblastoma	Scrapie (Chandler)	[15, 71, 85]
GT1	Mouse hippocampal neuron	Scrapie (Chandler)	[71, 108]
RK13	Rabbit kidney epithelial cell	Scrapie	[131]
SH-SY5Y	Human neurobrastoma	CJD	[58]
ScHB	Scrapie-infected hamster brain cell	Scrapie (Chandler)	[19]
SMB	Scrapie-infected mouse brain cell	Scrapie (Chandler)	[19]
NIH/3T3	Mouse fibroblast	Scrapie (22L)	[135]
L929	Mouse fibroblast	Scrapie [22L, ME7 and Chandler (RML)]	[135]
MNB	Mouse neuroblastoma	Scrapie (Chandler)	[85]
HpL3-4-PrP	Mouse hippocampal neuronal cell	Scrapie (Chandler)	Unpublished results (Sakudo A *et al.*)

CJD: Creutzfeld-Jakob.

only the CNS but also the placenta, lymphoreticular systems (see above), and muscles [10, 127]. As the use of prion-susceptible cell line N2a is virtually simple for culture, this cell line is the most extensively employed for prion infection studies, although its low post-infection titers and rapid attenuation of titers require improvement [15, 85]. Neurohypothalamic cell line GT1 displays higher PrP^C expression levels and is more susceptible to prion-infection than other cell lines [71, 108]. Although infection of human neuroblastoma cell line SH-SYS5 with CJD prions has been reported, reproducibility of this result has not been confirmed by other laboratories [58]. Therefore, cell culture systems for CJD and BSE prion infection have yet to be established.

USEFULLNESS OF CELL LINES FOR STUDYING PRION INFECTIONS

Transgenic mice have been used to analyze the specific amino acid residues and domains of PrP^C for prion infection and PrP^{Sc} accumulation in organs/tissues [5, 18, 27, 28, 78, 116, 120, 126]. In sheep, the incidence of natural scrapie is associated with polymorphisms of the PrP gene (*Prnp*), particularly those at codons 136, 154, and 171 [23], and arginine at position 171 in particular confers predominant resistance to scrapie [139]. Although the results obtained from these experiments provide important information on prion biology, the production of transgenic mice is time-consuming and expensive. Therefore, this experimental approach is not practical for detailed analysis of PrP domains and amino acid residues, which requires use of many types of PrP mutant.

Cell culture models designed for study of prion infection facilitate better understanding of the molecular mechanisms of PrP^{Sc} formation as well as the roles of the amino acid sequence and structural domains of PrP for conversion of PrP^C to PrP^{Sc} in a cell-autonomous fashion [43, 50, 132]. Hitherto, these types of studies, which have used persistently infected cell cultures [80, 93, 115] as *de novo* infections of cell cultures with prions, are restricted by a relatively low infection efficiency [86]. Cell lines susceptible to prions co-expressed with exogenous PrP are used for certain infection experiments [86, 108, 131, 135]; however, interference of endogenous PrP by exogenous PrP in terms of the pathogenicity of prion agents from other species has also been reported [87, 88, 115, 125]. Moreover, concomitant expression of heterogenous PrP species seems to inhibit prion infection, even if cell lines expressing undetectable PrP levels (e.g., rabbit kidney epithelial RK13 cells) are used [131]. Therefore, $Prnp^{-/-}$ cell lines lacking endogenous PrP may serve as models for analysis of the real domain of PrP^C required for prion pathogenicity without interfering with endogenous PrP by transfection of various deletion mutants of PrP following prion-infection [57].

Although most cell lines and primary cells express PrP^C [100], only a limited few are susceptible to prion-infection. Moreover, it is intriguing that both neuronal and non-neuronal cell line are susceptible to prion infection [121]. PrP^C appears to be not the sole determinant factor for prion susceptibility, and other cellular factors may be involved in efficient infections. Under certain conditions, loss of host factors for susceptibility to prion infection may occur [9, 54, 133]. Furthermore, most cell lines have restricted prion strain specificity, i.e., matching pairs between the host cell and prion strain.

Recent development of human embryonic stem cells offers the promise of replacement of damaged adult cells with normal cells in regenerative medicine [25], although the potential risk of infection of these cells with pathogens has been overlooked. Previously established cell lines, including human embryonic stem cell lines, should be examined to determine whether or not they have been contaminated with prions using sensitive diagnostic tests. Furthermore, we emphasize that research studies with newly created prion-susceptible human embryonic stem cell lines would contribute to elucidation of the infection mechanisms in prion-related diseases.

Finally, we would like to emphasize that the $Prnp^{-/-}$ cell line is reliable and useful for analysis of both PrP^{Sc} accumulation and PrP^C functions [59, 70, 102, 103–106]. PrP^C expression is required for prion infection [11, 14, 63, 83], while after prion infection, PrP^C-derived PrP^{Sc} is accumulated in organs/tissues, especially in the brain. Although elucidation of the functions of PrP^C is crucial in understanding the etiological mechanisms underlying prion diseases, these functions remain hitherto unclear in studies with $Prnp^{-/-}$ mice. In this review, we avoided redundant commentary on cell lines and PrP functions, as an extensive review has previously been documented [100].

CONCLUSION

Distribution of PrP^{Sc} depends on the prion strain and host species [52, 124]. Certain specific combinations of strain and host may lead to PrP^{Sc} induction/distribution in the blood and urine. Therefore, development of a reliable method that facilitates PrP^{Sc} detection in the blood and urine is urgently needed in terms of prion diagnosis and the safety of blood supplies in hospital wards.

Prion diseases can usually only be diagnosed by time-consuming methods such as ELISA, Western blotting, immunohistochemical analysis, and animal bioassays [30]. Recently, dramatic improvement has been achieved for prion-sensitive diagnostic methods, although further development of analytical methods to specifically and reliably detect prions is even more important with increasing emphasis on reducing prion-related risks in terms of public health and the safety of blood supplies.

Cell lines are valuable in analysis of the mechanisms of both PrP^{Sc} accumulation and PrP^C functions. If *Prnp* was reintroducted into $Prnp^{-/-}$ cells and the resulting cells were susceptible to certain prions, this cell line system would offer profound advantages over previous prion cell culture models because novel forms of PrP^{Sc} could be derived

entirely from exogenous PrP and appropriated for testing multiple artificial PrP molecules. Furthermore, it is noteworthy that PrP^{C}-to-PrP^{Sc} conversion is inhibited by concomitant expression of heterogenous PrP species. Absence of endogenous PrP in *Prnp*$^{-/-}$ cells could prevent the PrP^{C}-to-PrP^{Sc} inhibition. Moreover, study of PrP^{C} functions using the *Prnp*$^{-/-}$ cell line is gradually increasing, although the findings related to this subject from studies with *Prnp*$^{-/-}$ mice remain unclear. By introducing artificial PrP molecules to PrP-deficient cell lines, certain PrP portions critical for maintaining cell viability and increasing *de novo* synthesized prions may be elucidated. In addition, a combination of *Prnp*$^{-/-}$ cells and recently developed diagnostic methods, such as PMCA, would also contribute to understanding the mechanisms related to prion diseases.

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