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Development of a radioimmunoassay for screening of prion diseases

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Development of a radioimmunoassay for screening of prion diseases

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TABLE OF CONTENTS

	Page
ABBREVIATION LIST	iv
CHAPTER 1 LITERATURE REVIEW	
1.1 Prion	1
1.1.1 From disease to prion protein	1
1.1.2 The structure of prion protein	2
1.1.3 Replication and cytotoxicity of PrP^{SC}	5
1.1.4 Transmissible spongiform encephalopathy	8
1.2 Transmission Pathway of Prion Disease	13
1.2.1 Natural route of prion disease transmission	13
1.2.2 Accumulation of PrP^{SC} in lymphoid tissues	15
1.2.3 From peripheral tissues to CNS	18
1.3 Methods for Detection of Prion Disease	19
1.3.1 Bioassays	19
1.3.2 Prion-misfolding cyclic amplification	20
1.3.3 Quaking-induced conversion	21
1.3.4 Extraction of PrP^{SC} by stainless steel	23
1.3.5 Perspectives	23
1.4 References	24
CHAPTER 2 DEVELOPMENT OF A RADIOIMMUNOASSAY FOR SCREENING OF PRION DISEASES	
2.1 Abstract	38
2.2 Introduction	39
2.3 Materials and Methods	40
2.4 Results	44

2.5 Discussion 52

2.6 References 55

Abbreviation List

Abbreviation	Full name
ADAM10	A-disintegrin-and-metalloproteinase 10
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CD	Circular dichroism
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system
^{Ctm}PrP	Abnormal transmembrane prion protein
CV	Coefficient of variation
CWD	Chronic wasting disease
DCs	Dendritic cells
dpi	Day post-inoculation
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELSPOT	Enzyme-linked immunospot
ER	Endoplasmic reticulum
fCJD	Familial Creutzfeldt-Jakob disease
FFI	Fatal familial insomnia
FTIR	Fourier-transform infrared
GPI	Glycosylphosphatidylinositol
GSS	Gerstmann- Sträussler-Scheinker disease

M cells	Microfold cells
mAb	Monoclonal antibody
MES	2-(N-morpholino)ethanesulfonic acid
NAD^+	Nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
PEG	Polyethylene glycol
PMCA	Protein-misfolding cyclic amplification
PMSF	Phenylmethylsulfonylfluoride
PNS	Peripheral nervous system
$PrP_{143-153}$	Residue 143-153 of prion protein
PrP^C	Cellular prion protein
PrP^{SC}	Protease-resistant prion protein
QuIC	Quaking-induced conversion
recPrP	Recombinant prion protein
RIA	Radioimmunoassay
RT-QuIC	Real time quaking-induced conversion
sCJD	Sporadic Creutzfeldt-Jakob disease
TNF-α	Tumor-necrosis factor α
TNT	Tunneling nanotube
TPrP	Toxic prion protein
TSE	Transmissible spongiform encephalopathy
UV	Ultra-violet
vCJD	Variant Creutzfeldt-Jakob disease

CHAPTER I

LITERATURE REVIEW

1.1 Prion

1.1.1 From disease to prion protein

Scrapie, a prion disease in sheep, has been recognized over 250 years throughout the world. In 1914, scrapie was regarded as a muscle disease caused by a parasite infection [1]. After two decades, the transmission of scrapie was demonstrated, which led to the concept that etiological agent of scrapie was a virus [2, 3]. Because the disease progression is slow, it was named as the unconventional slow virus [4]. However, based on the findings of two studies, a virus or a viroid was ruled out being the pathogen of scrapie at that time. First, the infectivity of scrapie is not affected by exposure to the 245 nm ultra-violet (UV) irradiation that induces the polynucleotide damage [5]. Second, chemical or enzymatic modification of nucleic acids does not cause inactivation of scrapie agent [6]. Nevertheless, scrapie is inactivated by pretreatment with protein denature chemical agents or proteases, which is strong evidence that the scrapie agent contains protein. Also, in the phenyl-Sepharose column, the scrapie agent is difficult to be eluted without a detergent, which indicates it is a hydrophobic particle [7]. In 1982, a new term "prion" was proposed for scrapie agent by Dr. Stanley B. Prusiner, which was derived from the term "**pro**teinaceous **in**fectious" [6].

After pretreatment with proteinase K, the brain tissue from the scrapie-infected hamsters shows a band at 27-30 kD, designated as PrP^{27-30}, in a western blot assay,

but this protein is not found in health hamsters [8]. Through the purification of PrP^{27-30}, the N-terminal amino acid sequence of PrP^{27-30} was determined, giving a key for creating the cDNA clone and identifying its mRNA [9]. With cDNA probes, the PrP^{27-30} specific mRNA was found in the brain tissue from both the healthy and scrapie-infected hamsters [10]. In a southern blot study, PrP^{27-30}-related DNA sequences were detected in normal mice, hamsters and humans [11]. The findings suggested that PrP^{27-30} is a component of the normal protein in brain tissue, and it is encoded from a chromosomal gene. By using the same technique, PrP^{27-30}-related polynucleotide was not found in the purified scrapie agent, further confirming that the scrapie pathogen was not a virus or a viroid [11]. A complete cDNA clone and open reading frames were identified in hamsters in 1986, and *Prnp* gene was located on a single coding exon [12, 13]. This finding disproved the hypothesis that the protease-resistant prion protein, designated as PrP^{SC}, is created by alternative splicing from *Prnp* gene, but the mechanism for producing PrP^{SC} remained elusive. In a western blot study, cellular prion protein (PrP^{C}) and PrP^{SC} were distinguished by limited proteolysis [14]. In addition, the size of PrP^{C} is the same as PrP^{SC}, 33-35 kD, and both are only found in the cellular membrane fraction, which indicates a membrane protein.

1.1.2 The structure of prion protein

Through Fourier-transform infrared (FTIR) spectroscopy and circular dichroism (CD) spectrum, the secondary structure of PrP^{C} and PrP^{SC} were identified [15]. The content of PrP^{C} was 42% α-helix and 3% β-sheet, but PrP^{SC} had 30 % α-helix and

43% β-sheet. These results indicated that the major difference between PrP^{C} and PrP^{SC} is the protein secondary structure, β-sheet, which may capacitate the resistance to proteases. In an electron microscopy study, the rod-shape aggregates were formed by polymerization of PrP^{27-30}, which contained 54% β-sheet, but it was not detected in purified PrP^{C} or PrP^{SC} solution. The above findings suggested that β-sheet participates in the formation of aggregates. Due to the insolubility of PrP^{SC}, there is no high-resolution structure of PrP^{SC}, which impedes the understanding of the process of forming aggregates. Through simulation analysis of low-resolution projection map of PrP^{SC} and dimer structure of recombinant prion protein (recPrP), the β-sheet on PrP^{SC} was found to be responsible for intermolecular interaction and promotes the formation of PrP^{SC} trimers [16-19]. In addition, based on the dimer structure of recPrP, it was hypothesized that the formation of PrP^{SC} is due to the conformational change from α-helix to β-sheet [16].

The structure of PrP^{C} was further studied by X-ray crystallography and nuclear magnetic resonance (NMR) in recPrP [16, 20, 21]. The C-terminal of PrP is a globular structure consisting of three α-helices (residues 143-152, 171-191 and 199-221) and two antiparallel β-sheets (residues 127-129 and 166-168) (Figure 1). The N-terminal of PrP is unstructured, but the histidine-containing octarepeat (residue 51-90) binds with Cu^{2+}, which causes "folding" in this region [22]. In addition, a highly hydrophobic region (residue 112-130) makes PrP capable of forming an abnormal transmembrane structure, designated ^{Ctm}PrP, in endoplasmic reticulum (ER), and over-accumulation of ^{Ctm}PrP causes cell death [23]. There are three posttranslational modifications on PrP, including disulfide bond (between residues

179 & 214), N-linked glycosylation (residue 180 & 196) and glycosylphosphatidylinositol (GPI) anchor (residue 230-254). *In vitro* studies showed that expression of unglycosylated PrP^{C} or blockade of PrP^{C} glycosylation facilitates the infection of prion disease [24, 25]. It is likely that the N-linked oligosaccharides on PrP^{C} efficiently prevent the direct intermolecular interaction, which decreases the chance of conformational changes. In Creutzfeldt-Jakob disease (CJD) studies, a different type of CJD showed a distinct glycosylated pattern due to the different polysaccharides modification, which can be used to distinguish strains of CJD [26, 27]. An *in vitro* study showed that excessive recPrP on artificial plasma membrane induced the conformational change, which increased the content of β-sheet [28]. Amyloid plaques were found in the brain of scrapie-infected transgenic mice, expressing anchorless PrP, but with minimal clinical manifestations [29]. These results indicated that the GPI anchor is crucial for replication of PrP^{SC} and development of prion disease. Also, there is a possibility that accumulation of excessive PrP^{C} on the plasma membrane induces conformational changes and creates abnormal PrP.

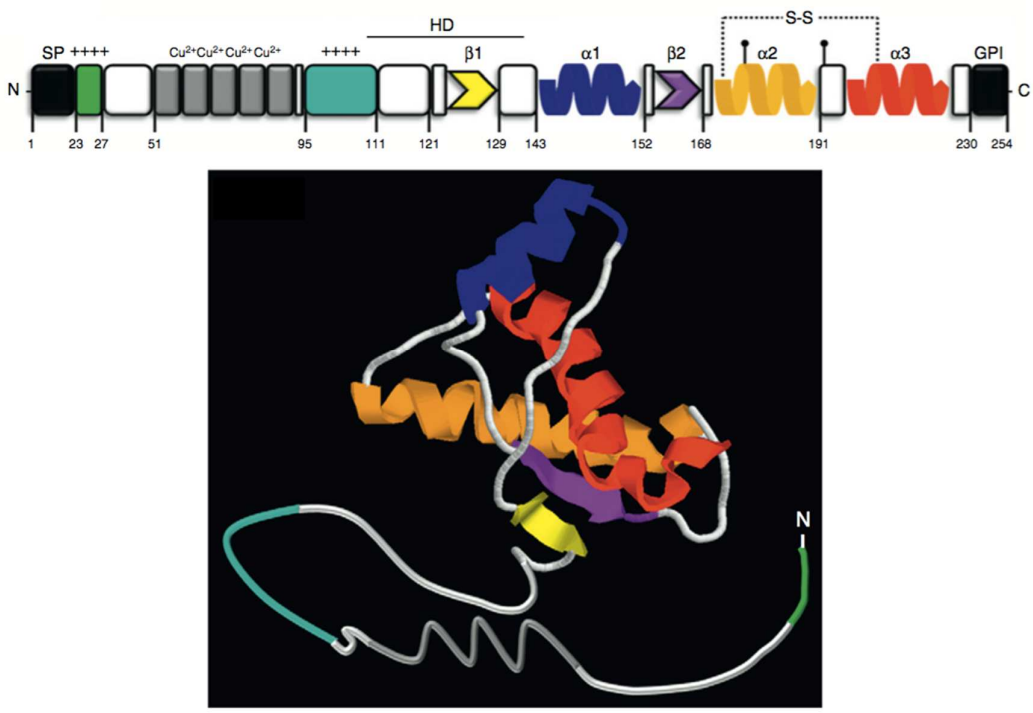


Figure 1. The amino acid sequence and structure of PrP^{C}. Residues correspond to the mouse sequence and the structure are based on the NMR analysis. ++++, polybasic region; Cu^{2+}, copper binding domain; SP, signal peptide; HD, hydrophobic domain; GPI, glycosylphosphatidylinositol. Reprinted from *Trends in Neurosciences*, 35(2), Biasini et al., Prion protein at the crossroads of physiology and disease, 92-103, Copyright (2012), with permission from Elsevier.

1.1.3 Replication and cytotoxicity of PrP^{SC}

Here, two models illustrate the replication of PrP^{SC}. *In vivo* studies indicated that the conformational change of PrP^{C} is through the formation of PrP^{C}/PrP^{SC} complex, and the process involve multiple intermolecular interactions [30-32]. However, because it is difficult to purify the PrP^{C}/PrP^{SC} complex, the mechanism of conformational conversion is still unclear. Based on this observation, the

"template-directed refolding" model is proposed. The intermolecular interactions overcome the energy barrier of conversion of PrP^{C} into PrP^{SC}, and eventually PrP^{C} turns itself into PrP^{SC} (Figure 2A). Another hypothesis is called "seeded nucleation" which suggests that PrP^{C} and PrP^{SC} are in a reversible equilibrium [33, 34]. In the normal state, the majority of PrP is in the PrP^{C} conformation, but in the diseased state, most of PrP shifts toward the PrP^{SC} conformation (Figure 2B). Subsequently, PrP^{SC} aggregates into a highly ordered oligomer that serves as a seed for a faster propagation, and eventually forms amyloid. The fragmentation of amyloid creates more seeds for recruiting more PrP^{SC}. In addition, the replication of PrP^{SC} is affected by the amount and amino acid sequence of PrP^{C}. There are 16 amino acid differences between the mouse PrP and the hamster PrP, and it could affect intermolecular interactions, causing a slower conformational conversion. The inoculation time for the development of prion disease in transgenic mice with expression of hamster PrP is prolonged, which supports the species barrier in prion disease [35, 36]. Ablation of PrP^{C} in mice decreases the propagation of PrP^{SC} and the disease progression, which further confirms that PrP^{C} is the source for replication of PrP^{SC} [37, 38].

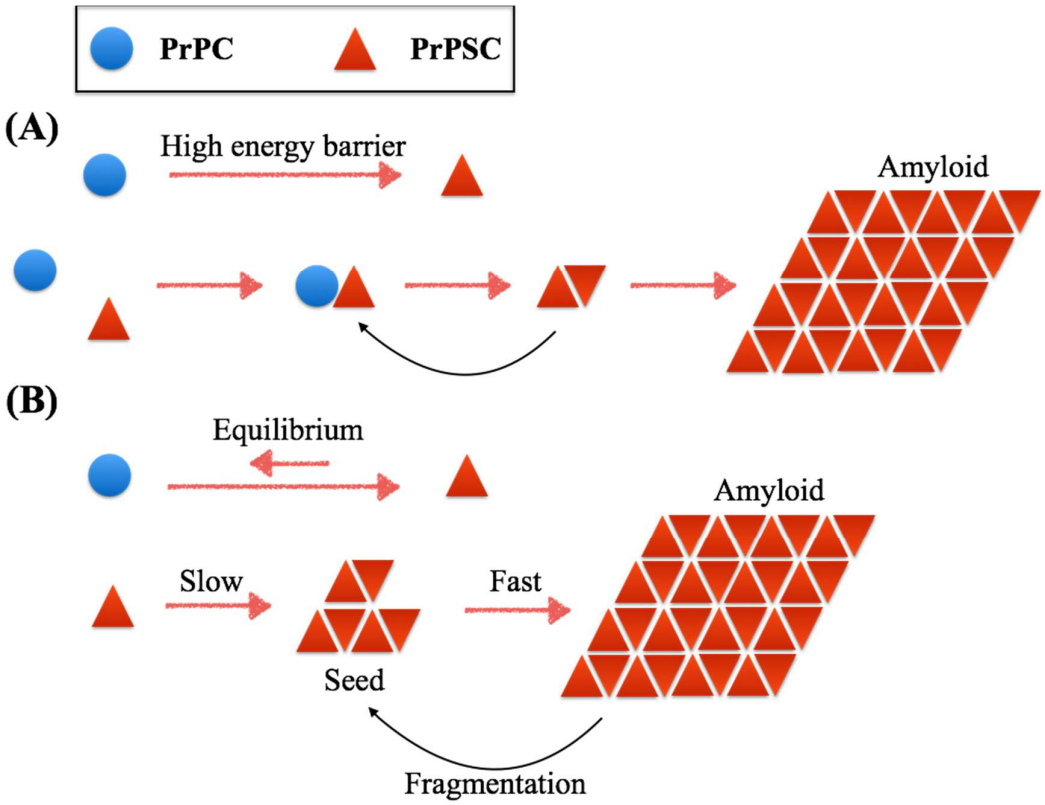


Figure 2. Models for the replication of PrP^{SC}. (A) The "template-directed refolding" model. The interactions between PrP^{C} and PrP^{SC} overcome the energy barrier of conformational conversion, and PrP^{C} turns itself into PrP^{SC}. Monomer PrP^{SC} is able to continually convert other PrP^{C}, and finally PrP^{SC} aggregates into amyloid. (B) The "seeded nucleation" model. PrP^{C} and PrP^{SC} are in a reversible equilibrium, and the formation of a highly ordered seed is slow. Once the seed is formed, it recruits more PrP^{SC} to aggregate into amyloid. The fragmentation of amyloid increases the amount of seeds that recruit more PrP^{SC} to form amyloid.

The question that needs to be addressed is how PrP^{SC} causes cytotoxicity and induces cell death. Purified PrP^{SC} from scrapie-infected mice causes the increase of intracellular Ca^{2+} concentration in N2A neuroblastoma cells, but it is prevented by

pretreatment with thapsigargin that depletes the ER Ca^{2+} stores [39]. The increase of intracellular Ca^{2+} concentration induces the calpain-caspase 12-mediated apoptosis, but the PrP^{SC}-induced cytotoxicity is alleviated by overexpression of Grp58, an ER chaperon [40]. These findings provide the evidence that PrP^{SC} causes ER stress and induces the caspase 12-mediated neuronal apoptosis in prion disease. In addition, the misfolded full-length recPrP shows high neurotoxicity *in vitro* and *in vivo*, known as toxic prion protein (TPrP), which is in a monomeric α-helix form [41]. TPrP causes autophagy and apoptosis that are similar to the observation in the brain of prion-infected animals. The further study indicated that TPrP causes reduction of intracellular nicotinamide adenine dinucleotide (NAD^+) in neuronal cells, which decreases the production of ATP and eventually induces cells death [42]. Although TPrP is an artificial PrP species, it demonstrates a possibility that highly neurotoxic PrP monomer is created during the conformational conversion of PrP^{C} to PrP^{SC}. In addition, this PrP monomer could directly cause neuron death without the process of aggregation.

1.1.4 Transmissible spongiform encephalopathy

Prion disease is characterized as a neurodegenerative disorder, which causes clinical symptoms including dysfunction of cognition and motor. In brain pathology, amyloid plaques, formed by aggregates of PrP^{SC}, are found in animals with prion disease. Also, prion disease is regarded as the propagation of infectious prions, which causes the death of neuronal cells and forms tiny holes in the cerebral cortex. Thus, prion disease is also known as transmissible spongiform encephalopathy (TSE).

Prion diseases have been diagnosed in several species, including scrapie in sheep, Kuru and CJD in humans, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in deer (Table 1).

Table 1. Summary of prion diseases in different species.

Disease	Host Species	Route of transmission
Kuru	Human	Ritualistic cannibalism
Sporadic CJD	Human	Unknown etiology
Iatrogenic CJD	Human	Accidental exposure to CJD-contaminated tissues
Inherited CJD	Human	Mutations in *Prnp* gene
Variant CJD	Human	Ingestion of BSE-contaminated food
Scrapie	Sheep, goat and mouflon	Horizontal and possibly vertical transmission
BSE	Cattle	Ingestion of BSE-contaminated meat and bone
CWD	Mule deer, white-tailed deer, Rocky mountain elk and moose	Horizontal and possibly vertical transmission

The first scrapie case was described in 1738, and the diseased sheep showed symptoms including aggression, tremors, incoordination and scratching [43]. Scrapie has been found around the world, except in Australia and New Zealand, and this disease can only be transmitted within the flock. In addition, PrP^{SC} was detected

in milk, feces and saliva from scrapie-infected sheep, which indicated scrapie may be transmitted through these scrapie-contaminated agents [44-46]. In 1998, five cases of atypical scrapie were described in Norway, which was named as Nor98 [47]. In these animals, PrP^{SC} was only found in the cortex of the cerebrum and the cerebellum, but not in the lymphoid tissue. Although atypical scrapie has been found in many countries, including New Zealand; in each case, only a few animals were diagnosed as the prion disease in the same cluster [48]. These observations support that atypical scrapie is poorly transmitted, but the pathogenesis of this disease remains unclear.

In 1980, the CWD of deer and elk was identified in Colorado and Wyoming, and symptoms included emaciation, excess rumen fluid and spongiform transformation in the central nervous system (CNS) [49]. To date, CWD has only been found in the United States, Canada and South Korea. CWD can be transmitted through saliva, blood and feces to other healthy deer, but the disease origin is still unclear [50-52]. The major concern is whether CWD can be transmitted to other species, especially humans. By oral inoculation, CWD has not been transmitted to other species outside of the cervid family [53]. However, transgenic mice with expression of elk or deer PrP^{C} develop prion disease after inoculation with the brain from CWD-infected animals, but the disease is not found in transgenic mice expressing human, ovine or bovine PrP^{C} [54, 55]. An *in vitro* study showed that CWD-associated PrP^{SC} was less efficient to convert bovine or human recPrP into PrP^{SC}, comparing with cervid recPrP [56]. These studies indicated there is a strong species barrier for CWD transmission. In addition, no large-scale of human prion disease has been reported in Colorado and Wyoming for the past three decades, which supports that CWD is not transmitted to humans [57].

In 1986, an epidemic of spongiform encephalopathy was identified in cattle in the United Kingdom, known as BSE or "mad cow" [58]. BSE is classified as a prion disease, because PrP^{SC} is found in the brain from diseased cattle [59]. More than 35,000 cattle were diagnosed in 1992, which reached the peak of BSE infection (Figure 3A). The large-scale outbreak of BSE is attributed to feeding of BSE-contaminated meat and bone as supplement to cattle [60]. In 2003, another peak of BSE cases occurred mainly in France and Ireland, and this outbreak of BSE was well-controlled. In last 5 years, no more than 30 cases of BSE per year were reported worldwide. Although the epidemic of BES is well-controlled, it has still not been eradicated. Can BSE be transmitted to humans? An *in vivo* study demonstrated that non-human primates receiving oral inoculation of the brain from BSE-infected cattle developed prion disease [61]. The PrP^{SC} from BSE-infected and vCJD-infected mice showed undistinguishable glycosylated pattern, suggesting that BSE and vCJD are caused by the same prion strain [62]. In addition, based on the cases of BSE and variant CJD (vCJD) in the UK, it shows that the outbreak of vCJD correlates with the prevalence of BSE in 1992 (Figure 3B). These pieces of evidence support the notion that BSE can be transmitted to humans and develop vCJD.

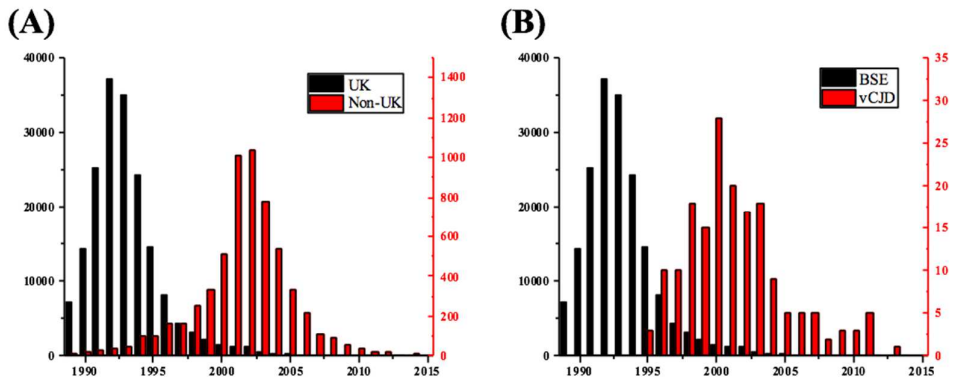


Figure 3. Annual cases of bovine spongiform encephalopathy (BSE) reported worldwide and variant Creutzfeldt-Jakob disease (vCJD) in the United Kingdom (UK). (A) The cases of BSE in the UK (black) and in countries excluding the UK (red). The peak in early 2000s majorly occurred in France and Ireland. Data are updated on April 2015 (http://www.cjd.ed.ac.uk). (B) The cases of BSE (black) and vCJD (red) in the UK. Data are updated in April 2015 (http://www.oie.int).

Kuru, a human transmissible spongiform encephalopathy, was reported in the region of Papua New Guinea in 1950s, and transmission of disease was imputed by ritualistic cannibalism [63]. CJD is classified into four types, including variant, iatrogenic, inherited and sporadic. Variant CJD was first identified in 1996 and is linked to BSE. Iatrogenic CJD is an accidental transmission from other CJD patients during surgical procedures [64]. Also, several cases were from receiving human growth hormone or pituitary gonadotropins derived from CJD patients [65-68]. Inherited CJD is due to alteration of *Prnp* gene, which includes familial CJD (fCJD), Gerstmann- Sträussler-Scheinker disease (GSS) and fatal familial insomnia (FFI). fCJD is associated with the point mutation at codon 145 of *Prnp* gene that creates the

premature stop codon, which generates the deposition of PrP and Tau protein [69, 70]. Also, fCJD is characterized by rapid progression of dementia. GSS is a rare inherited and slow progressive disease, which is caused by the mutation in codons P102L and F198S of *Prnp* gene [71]. The point mutation of D178N of *Prnp* gene causes FFI which is characterized by refractory insomnia and dysautonomia [72]. Sporadic CJD (sCJD) accounts for 85% of human prion diseases. In sCJD patients, there is no mutant *Prnp* allele, nor evidence for contact with infected patients or animals. The polymorphism of codon 129 of *Prnp* gene (methionine/valine) has a strong correlation with sCJD. Clinical studies showed 21 out of 22 sCJD cases were homozygous at codon 129 of *Prnp* gene [73]. However, the pathogenesis of sCJD remains elusive.

1.2 Transmission Pathway of Prion Disease

1.2.1 Natural route of prion disease transmission

The cases of Kuru and vCJD illustrate that the natural route of the prion disease pathogenesis is through oral exposure. This transmission route is confirmed by the following studies. Oral inoculation of brain homogenates from animals infected with scrapie or BSE caused the development of the prion disease in sheep and mice [74, 75]. Also, CWD can be transmitted via oral route between deer [76, 77].

How is PrP^{SC} transported from the lumen of the gastrointestinal tract to lymphoid tissues? Microfold cells (M cells) take up particulate antigens from the gut lumen and transport it to Peyer's patches to stimulate the adaptive immune

response. In order to study the transportation of PrP^{SC} by M cells, a device was designed that contained a filter seeded with Caco-2 cells and Raji B cells, placed on another side [78]. The B cells can migrate through the filter and promote Caco-2 cells to differentiate into M cells. This study showed that the M cells containing cocultures transported the prion protein to the basolateral chamber, but Caco-2 cultures did not transfer prion to the other side. Mice with depletion of M cells decrease the early accumulation of PrP^{SC} in Peyer's patches and further neuroinvasion [79]. Both studies indicated that M cells are important for transporting the prion protein from the gut lumen to Peyer's patches (Figure 4).

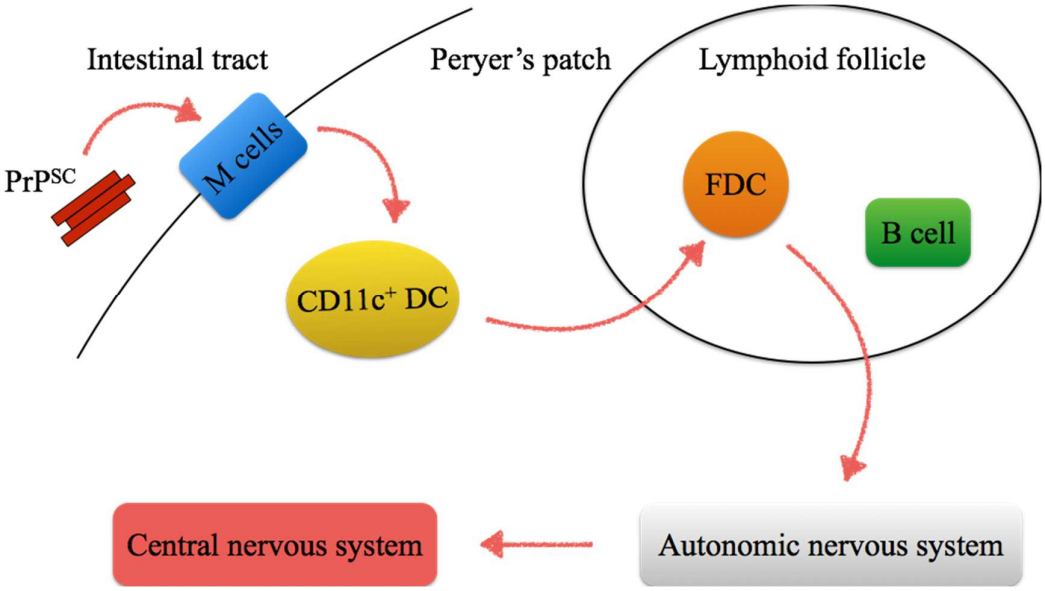


Figure 4. Summary of the oral route of PrP^{SC} transmission. PrP^{SC} is transported by microfold cells (M cells) from gut lumen into the Peyer's patch. $CD11c^{+}$ dendritic cells (DCs) receive PrP^{SC} from M cells and migrate to lymphoid follicle where PrP^{SC} is delivered to FDC. PrP^{SC} replicates on the plasma membrane of follicular dendritic cells (FDCs), and PrP^{SC} undergo a retrograde neuroinvasion

through afferent autonomic nervous system; eventually, PrP^{SC} reaches central nervous system.

Who receives PrP^{SC} from M cells and spreads it to lymphoid tissues? The rats treated with scrapie-associated fibrils, highly infective aggregates of PrP^{SC}, showed that migratory dendritic cells (DCs) were a carrier of PrP^{SC}, but not T or B lymphocytes [80]. Exposing $CD11c^+$ myeloid DCs to scrapie infected GT1-1 cells, $CD11c^+$ DCs engulfed infected GT1-1 cells, but PrP^{SC} was degraded by the cysteine protease [81, 82]. In an oral inoculation study using the brain homogenate from scrapie-infect animals, $CD11c^+$ DC-depleted mice showed a blockade of prion protein accumulated in lymphoid tissues and a decrease of susceptibility to the disease [83]. Although previous studies showed that PrP^{SC} was degraded in $CD11c^+$ DCs, the *in vivo* study indicated $CD11c^+$ DCs play an important role in spreading PrP^{SC}.

1.2.2 Accumulation of PrP^{SC} in lymphoid tissues

In CJD-infected mice, CWD deer and scrapie sheep, accumulation of PrP^{SC} was detected in lymphoid tissues, e.g., lymph nodes, spleen, palatine tonsils and Peyer's patches in the early stage of disease progression [76, 84-86]. The results of an immunohistochemical study indicated that two kinds of cells highly expressing PrP^{C} in lymphatic system are follicular dendrite cells (FDCs) and DCs [87]. Confocal microscopy analysis showed the colocalization of FDCs and PrP^{C} in uninfected mouse spleen tissue [86]. FDCs are long-lived and non-migratory cells in primary and secondary follicles. Also, FDCs provide long-lasting intact antigens for

receptors on B cells in the germinal center. The immuno-electron microscopy study showed accumulation of PrP^{SC} was on the FDCs plasma membrane and intracellular space [88]. This report also showed that tangible-body macrophages received PrP^{SC} from FDCs, which facilitated the transportation of PrP^{SC} in the lymph system. There are two different ways for FDCs to hold PrP^{SC} on cell surface (Figure 5). First, the PrP^{C} on the plasmas membrane of FDCs interacts with PrP^{sc} to form an oligomer. Second, complement receptors (CD21 and CD35) on the surface of FDCs bind to complement-PrP^{SC} complexes. The susceptibility to the prion disease from peripheral infection was reduced in both C3/C1q$^{-/-}$ mice and CD21/CD35$^{-/-}$ mice [89-91].

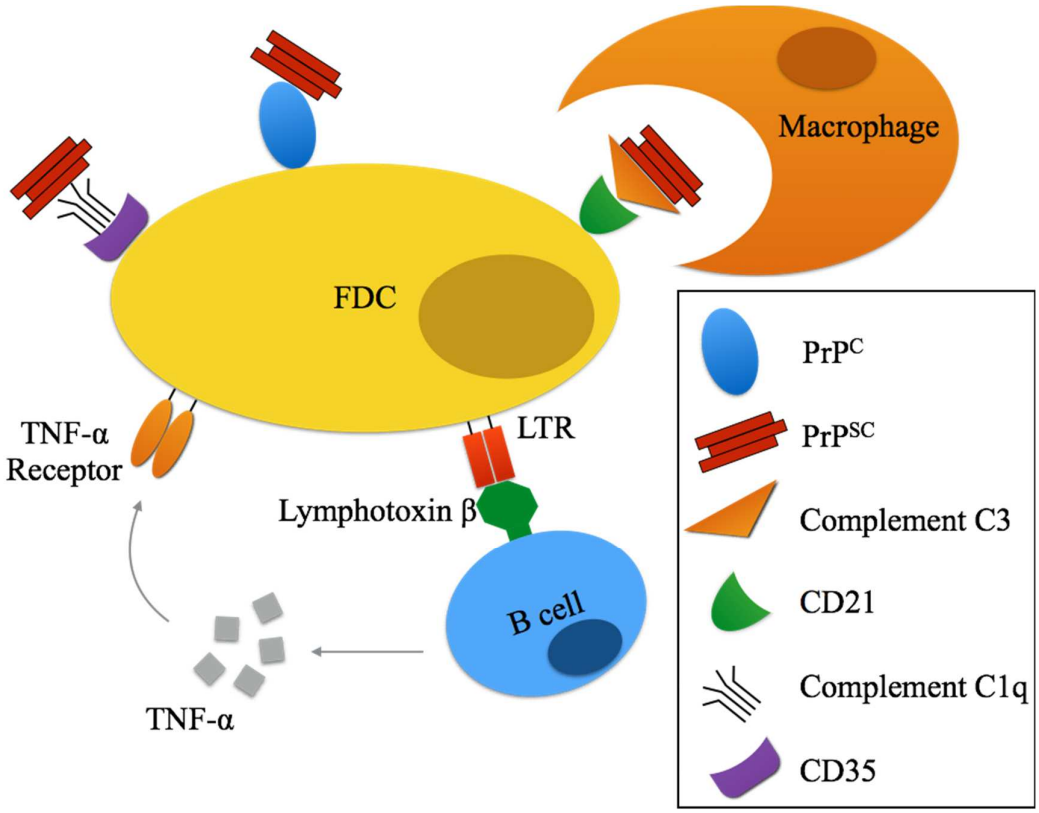


Figure 5. Maturation of FDC and PrP^{SC} presenting on FDC. PrP^{SC} is presented on FDC through PrP^{C}, complement C1q-CD35 and complement C3-CD21. The

presenting PrP^{SC} is engulfed by macrophages. The maturation of follicular dendritic cell (FDC) is achieved by B cell-dependent tumor-necrosis factor α (TNF-α) and lymphotoxin β in secondary lymphoid follicles.

Although PrP^{C} expression on B cells is not necessary and not sufficient for PrP^{SC} propagation [92, 93], B cells play an important role in promoting the maturation of FDCs through tumor-necrosis factor α (TNF-α) and lymphotoxin β (Figure 5). *In vivo* studies showed that TNF-α-knockout mice were resistant to peripheral prion infection [94, 95]. Blockade of TNF-α receptors by antibody showed the temporary inhibition of PrP^{SC} spread [96]. In peripheral inoculation studies, impedance of the lymphotoxin β signaling pathway reduced the susceptibility of the prion disease and decreased the accumulation of PrP^{SC} in the spleen [97-99]. However, through intracerebral inoculation, it did not change prion disease pathogenesis [100]. These findings further supported that matured FDCs are the crucial player in propagating PrP^{SC} from peripheral tissues to CNS, but other immune characteristics, e.g., B lymphocytes and complement components, are also required. Although suppression of FDCs maturation could reduce the spread of PrP^{SC}, it was only efficient during the early stage of the disease progression [101]. Numerous autonomic nerves are located in or near secondary lymphoid tissues, and the accumulated PrP^{SC} undergoes a retrograde neuroinvasion through these nerves to reach CNS.

1.2.3 From peripheral tissues to central nerve system

In hamsters infected with the scrapie protein orally, PrP^{SC} was found in myenteric and submucosal plexuses on day 69 post-inoculation (dpi). In addition, it was found in mesenteric lymph nodes, mucosal lymphoid follicles and Peyer's patches [102]. At 76 dpi, PrP^{SC} was abundant in the ganglia of the enteric nervous system and the associated nerve fibers. In BSE-infected cattle, PrP^{SC} was found in the vagus nerve and coeliac mesenteric ganglion complex [103]. In vCJD patients, immunochemical analysis showed that PrP^{SC} was accumulated at celiac ganglia and stellate ganglia [104]. Animals receiving oral inoculation of brain homogenate from animals with PrP^{CS} showed that PrP^{SC} appeared in the sympathetic nerve system [105-107]. Chemical sympathectomy in mice was achieved by injection of 6-hydroxydopamine and anti-nerve growth factor antibody, these mice showed a blockade or delay of prion disease progression [108]. On the other hand, keratin 14-nerve growth factor transgenic mice, which had hyperinnervated sympathetic nerves at lymphoid tissues, showed higher susceptibility to the prion disease. Above studies demonstrated that sympathetic nerves, parasympathetic nerves and associated fibers were possible routes for transferring PrP^{SC} from peripheral tissues to CNS.

How do cells transfer pathogenic prion protein to other cells? Although the mechanism details are not fully understood, several hypotheses have been proposed. Non-infected cells release PrP^{C} into culture medium via membrane vesicles; similar results were found in PrP^{SC}-infected cells in which PrP^{SC} was released into medium through exosome-like vesicles [109]. Tunneling nanotube (TNT) is a thin tube used by cells to communicate with other cells over a long distance. In mouse neuronal

cells, membranous and endogenous PrP^{SC} are transferred from infected cells to naive cells by TNTs. Also, bone marrow-derived DCs can interact with the primary neuron and transfer PrP^{SC} by TNTs *in vitro* [110]. When analyzing non-fibrillar particles of the scrapie-infected hamster brain, it was found that these particles had 14-28 prion protein molecules, being the most efficient seed for the prion disease [111]. Based on this evidence, a free-floating PrP^{SC} model was proposed. Once pathogenic PrP reaches the spinal cord via the peripheral nerve system, it traverses in a retrograde direction to brain and forms the aggregate.

1.3 Methods for Detection of Prion Disease

The traditional methods to diagnose prion disease are through post-mortem immunohistochemical and neuropathological examinations, and they are still the most reliable assays for prion disease [112]. In recent years, several assays were developed for preclinical diagnosis of prion diseases using accessible body fluids, such as plasma and saliva.

1.3.1 Bioassays

In 1982, the animal bioassay of PrP^{SC} was established, and Syrian hamsters were used in this assay [113]. Syrian hamster is highly susceptible to prion disease through intracerebral inoculation of the brain tissue from scrapie-infected sheep. The time from inoculation to onset of symptoms or to death is inversely proportional to the dose of intracerebral injection of the scrapie-infected brain homogenate. However, this assay is time-consuming, costly and imprecise to determine the prion infectivity in brain sample. An improved bioassay was developed by using N2a

neuroblastoma cells that are susceptible to PrP^{SC} from scrapie-infected mice [114]. N2a cells are transferred to the membranes of enzyme-linked immunospot (ELSPOT) plates, and they go through lysis, proteinase K digestion and detection by PrP antibody. The number of PrP^{SC}-infected colonies is strongly correlated with the dose of the brain homogenate from scrapie-infected mice. Comparing with the hamster assay, N2a cells assay is ten times faster and less expensive.

1.3.2 Protein-misfolding cyclic amplification

According to the "seeded nucleation" model, the method of amplification of misfolded prion protein was established, known as protein-misfolding cyclic amplification (PMCA) [115]. PrP^{SC} from scrapie-infected hamster brain serves as seeds, and the raw material, PrP^{C}, is provided by health hamster brain. The amplifying reaction is accelerated by sonication that breaks up aggregates and produces more seeds for PrP conversion. Through western blot analysis, concentrations of PrP^{SC} in samples are estimated. After five PMCA cycles, more than 97% of PrP^{SC} becomes the newly converted protein. In addition, the amplified PrP^{SC} shows infectivity in hamsters [116]. Raw materials for PMCA are not only provided by brain homogenate, but also by hamster recPrP that decreases the sensitivity of PrP^{SC} assay [117]. The PMCA assay is widely used for different samples from animals with prion disease, including brain, blood, feces, urine and milk [118-121]. In scrapie-infected hamsters, PrP^{SC} concentrations are 2.3×10^{-5}g/g in brain, 2.0×10^{-11}g/g in spleen and 1.3×10^{-14}g/ml in plasma [122].

Although PrP^{SC} can be detected in blood samples by PMCA, it is still problematic. In order to amplify the PrP^{SC} from blood, more PMCA cycles are repeated over several days, which increases false positive results. Surround optical fiber immunoassay (SOFIA) is comprised of a set of monoclonal antibodies and the comprehensive high-energy fluorescence emission is measured. In the prototype of SOFIA, extraction of PrP^{SC} from the brain sample is achieved by digestion with proteinase K, and the detection limit is around 10^{-17} g of PrP^{SC} from scrapie-infected hamster brains [123, 124]. When limited serial PMCA and SOFIA are combined, it ameliorates the problem of high false positives and PrP^{SC} is detected in proteinase K-untreated plasma samples from scrapie-infected sheep and CWD-infected white-tailed deer [125]. However, due to the non-standardized instrument in SOFIA, this assay is difficult to be widely used for PrP^{SC} detection.

1.3.3 Quaking-induced conversion

Samples may receive different vibrational energy in PMCA, resulting from tube position, tube construction and type of sonicator. These factors make the process of sonication difficult to standardize, and affect the consistency of PMCA assay. In order to ameliorate the problem of sonication, periodic shaking substitutes the process of sonication in a new technique known as quaking-induced conversion (QuIC) [126]. Through the QuIC assay, samples, such as brain tissue and cerebral spinal fluid (CSF), from vCJD patients or scrapie-infected sheep were discriminated within 1-2 days [127]. Thioflavin T, a fluorescent emission dye, binds to the β-sheet-rich structure, such as amyloid aggregates [128, 129]. The amount of PrP^{SC} is quantified by

measuring the fluorescent intensity instead of the western blot analysis. This method provides the real-time information of PrP^{SC} conversion; therefore, it is named real-time QuIC (RT-QuIC) [130]. PrP^{SC} in brain tissue, CSF and nasal fluids were detected by RT-QuIC, but it cannot be used to analyze blood samples due to the low sensitivity [130-132]. Monoclonal 15B3 antibody is the PrP^{SC} conformational-specific antibody, and its epitope corresponds to residues 142-148, 162-170 and 214-226 of human PrP [133]. Through combination of the PrP^{SC} immunoprecipitation by 15B3 mAb and RT-QuIC, plasma or serum samples from scrapie-infected hamster were distinguished from health hamster (Figure 6) [134].

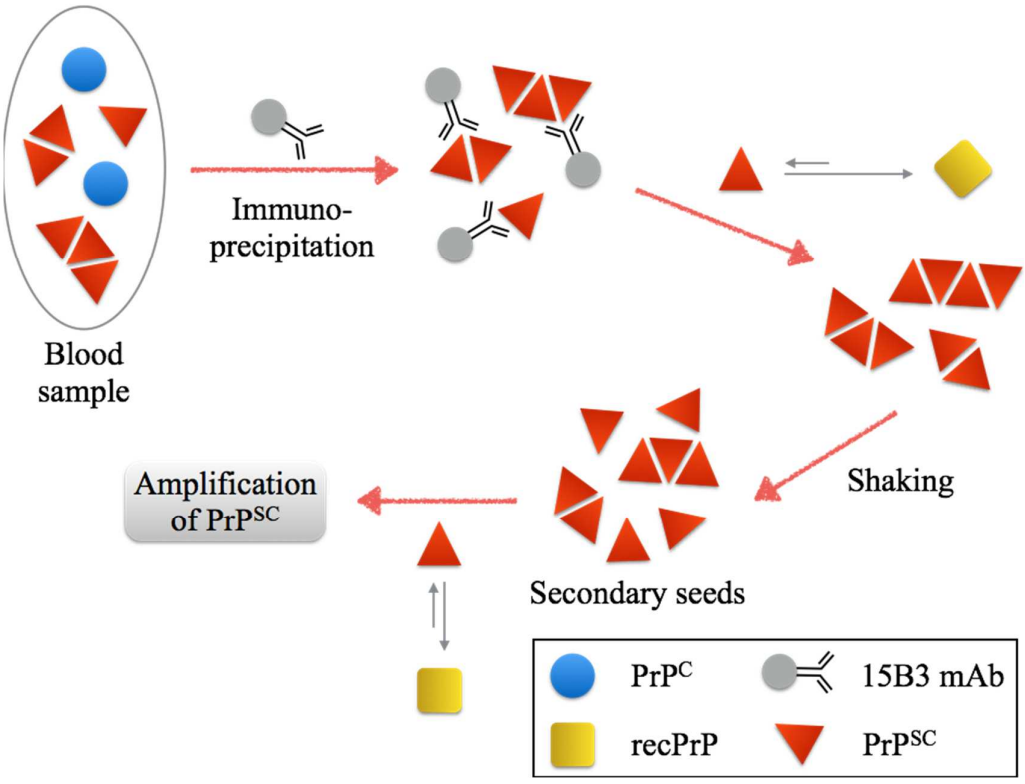


Figure 6. Mechanism of using immunoprecipitation and QuIC to detect PrP^{SC}. PrP^{SC} is pulled out from blood samples by 15B3 mAb, and the recPrP is added. Based on the "seed nucleation" model, recPrP is slowly converted into PrP^{SC} and

aggregates with PrP^{SC}. Shaking the tube causes the fragmentation of PrP^{SC} aggregates which produce more seeds known as secondary seeds. More seeds will provide a dock for newly converted PrP^{SC} which promotes the production of PrP^{SC}.

1.3.4 Extraction of PrP^{SC} by stainless steel

In addition to the PrP^{SC} immunoprecipitation method, stainless steel is also used to extract PrP^{SC} from blood samples. The study of iatrogenic CJD showed PrP^{SC} was transmitted through contaminated stainless steel surgical instruments [64, 135]. Further study showed that PrP^{SC} bond to nickel and molybdenum powder with high affinity [136]. Detection of PrP^{SC} in blood samples can be achieved by extracting PrP^{SC} with particles of stainless steel and measured by PrP enzyme-linked immunosorbent assay (ELISA) [137]. The blood samples from vCJD patients were distinguished by this assay [138]. More importantly, this method does not require amplification of PrP^{SC} through PMCA or QuIC, so it reduces the rate of false positive. However, this method showed 28.6% false negative due to low amount of PrP^{SC} in the sample [138].

1.3.5 Perspectives

Due to low solubility, the concentration of PrP^{SC} maybe rather low in body fluids. Although PrP^{SC} can be detected through the process of amplification or extraction, these assays showed a large number of false positives and false negatives. To avoid these false results, novel methods are needed to analyze body fluids, such as plasma to help with diagnosis of prion diseases. Based on the concept of PrP^{SC}

replication, PrP^{C} is converted into PrP^{SC} during the progression of prion disease. Thus, it is hypothesized that PrP^{C} is diminished in animals with prion disease. Here, we developed a radioimmunoassay to detect PrP^{C} in plasma. It is hope that this assay will be useful in the diagnosis of scrapie and CWD.

1.4 References

1. M'Gowan, J.P., *Investigation into the disease of sheep called " Scrapie": with especial reference to its association with sarcosporidiosis*. 1914: Blackwood.
2. Cuille, J. and P.L. Chelle, *Experimental transmission of trembling to the goat.* CR Seances Acad Sci, 1939. **208**: p. 1058-1060.
3. Wilson, D.R., R.D. Anderson, and W. Smith, *Studies in scrapie.* J Comp Pathol Ther, 1950. **60**(0): p. 267-IN12.
4. Merz, P.A., et al., *Infection-specific particle from the unconventional slow virus diseases.* Science, 1984. **225**(4660): p. 437-440.
5. Alper, T., et al., *Does the Agent of Scrapie Replicate without Nucleic Acid ?* Nature, 1967. **214**(5090): p. 764-766.
6. Prusiner, S.B., *Novel proteinaceous infectious particles cause scrapie.* Science, 1982. **216**(4542): p. 136-44.
7. Prusiner, S.B., et al., *Scrapie agent contains a hydrophobic protein.* Proc Natl Acad Sci U S A, 1981. **78**(11): p. 6675-9.
8. Bolton, D.C., M.P. McKinley, and S.B. Prusiner, *Identification of a protein that purifies with the scrapie prion.* Science, 1982. **218**(4579): p. 1309-11.
9. Prusiner, S.B., et al., *Purification and structural studies of a major scrapie prion protein.* Cell, 1984. **38**(1): p. 127-134.

10. Chesebro, B., et al., *Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain.* Nature, 1985. **315**(6017): p. 331-333.

11. Oesch, B., et al., *A cellular gene encodes scrapie PrP 27-30 protein.* Cell, 1985. **40**(4): p. 735-746.

12. Locht, C., et al., *Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with the scrapie agent.* Proc Natl Acad Sci U S A, 1986. **83**(17): p. 6372-6.

13. Basler, K., et al., *Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene.* Cell, 1986. **46**(3): p. 417-28.

14. Meyer, R.K., et al., *Separation and properties of cellular and scrapie prion proteins.* Proc Natl Acad Sci U S A, 1986. **83**(8): p. 2310-2314.

15. Pan, K.M., et al., *Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 10962-10966.

16. Knaus, K.J., et al., *Crystal structure of the human prion protein reveals a mechanism for oligomerization.* Nat Struct Mol Biol, 2001. **8**(9): p. 770-774.

17. Wille, H., et al., *Structural studies of the scrapie prion protein by electron crystallography.* Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3563-8.

18. DeMarco, M.L. and V. Daggett, *From conversion to aggregation: Protofibril formation of the prion protein.* Proc Natl Acad Sci U S A, 2004. **101**(8): p. 2293-2298.

19. Govaerts, C., et al., *Evidence for assembly of prions with left-handed β-helices into trimers.* Proc Natl Acad Sci U S A, 2004. **101**(22): p. 8342-8347.

20. Riek, R., et al., *NMR structure of the mouse prion protein domain PrP(121-231).* Nature, 1996. **382**(6587): p. 180-2.

21. Hornemann, S., et al., *Recombinant full-length murine prion protein, mPrP(23-231): purification and spectroscopic characterization.* FEBS Lett, 1997. **413**(2): p. 277-81.

22. Stockel, J., et al., *Prion protein selectively binds copper(II) ions.* Biochemistry, 1998. **37**(20): p. 7185-93.

23. Hegde, R.S., et al., *A transmembrane form of the prion protein in neurodegenerative disease.* Science, 1998. **279**(5352): p. 827-34.

24. Lehmann, S. and D.A. Harris, *Blockade of glycosylation promotes acquisition of scrapie-like properties by the prion protein in cultured cells.* J Biol Chem, 1997. **272**(34): p. 21479-87.

25. Korth, C., K. Kaneko, and S.B. Prusiner, *Expression of unglycosylated mutated prion protein facilitates PrP(Sc) formation in neuroblastoma cells infected with different prion strains.* J Gen Virol, 2000. **81**(Pt 10): p. 2555-63.

26. Collinge, J., et al., *Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD.* Nature, 1996. **383**(6602): p. 685-90.

27. Parchi, P., et al., *Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects.* Ann Neurol, 1999. **46**(2): p. 224-33.

28. Elfrink, K., et al., *Structural changes of membrane-anchored native PrPC.* Proc Natl Acad Sci U S A, 2008. **105**(31): p. 10815-10819.

29. Chesebro, B., et al., *Anchorless prion protein results in infectious amyloid disease without clinical scrapie.* Science, 2005. **308**(5727): p. 1435-9.

30. Prusiner, S.B., et al., *Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication.* Cell, 1990. **63**(4): p. 673-86.

31. Horiuchi, M., et al., *Interactions between heterologous forms of prion protein: binding, inhibition of conversion, and species barriers.* Proc Natl Acad Sci U S A, 2000. **97**(11): p. 5836-41.

32. Meier, P., et al., *Soluble dimeric prion protein binds PrP(Sc) in vivo and antagonizes prion disease.* Cell, 2003. **113**(1): p. 49-60.

33. Jarrett, J.T. and P.T. Lansbury, Jr., *Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie?* Cell, 1993. **73**(6): p. 1055-8.

34. Aguzzi, A. and M. Polymenidou, *Mammalian Prion Biology: One Century of Evolving Concepts.* Cell, 2004. **116**(2): p. 313-327.

35. Scott, M., et al., *Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques.* Cell, 1989. **59**(5): p. 847-57.

36. Moore, R.A., I. Vorberg, and S.A. Priola, *Species barriers in prion diseases--brief review.* Arch Virol Suppl, 2005(19): p. 187-202.

37. Prusiner, S.B., et al., *Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies.* Proc Natl Acad Sci U S A, 1993. **90**(22): p. 10608-10612.

38. Bueler, H., et al., *Mice devoid of PrP are resistant to scrapie.* Cell, 1993. **73**(7): p. 1339-47.

39. Hetz, C., et al., *Caspase-12 and endoplasmic reticulum stress mediate neurotoxicity of pathological prion protein.* The EMBO Journal, 2003. **22**(20): p. 5435-5445.

40. Hetz, C., et al., *The disulfide isomerase Grp58 is a protective factor against prion neurotoxicity.* J Neurosci, 2005. **25**(11): p. 2793-802.

41. Zhou, M., et al., *Highly neurotoxic monomeric α-helical prion protein.* Proc Natl Acad Sci U S A, 2012. **109**(8): p. 3113-3118.

42. Zhou, M., et al., *Neuronal death induced by misfolded prion protein is due to NAD+ depletion and can be relieved in vitro and in vivo by NAD+ replenishment.* Brain, 2015. **138**(Pt 4): p. 992-1008.

43. Parry, H.B. and D.R. Oppenheimer, *Scrapie disease in sheep. Historical, clinical, epidemiological, pathological and practical aspects of the natural disease*. 1983: Academic Press.

44. Terry, L.A., et al., *Detection of prions in the faeces of sheep naturally infected with classical scrapie.* Vet Res, 2011. **42**: p. 65.

45. Tamguney, G., et al., *Salivary prions in sheep and deer.* Prion, 2012. **6**(1): p. 52-61.

46. Konold, T., et al., *Evidence of effective scrapie transmission via colostrum and milk in sheep.* BMC Vet Res, 2013. **9**: p. 99.

47. Benestad, S.L., et al., *Cases of scrapie with unusual features in Norway and designation of a new type, Nor98.* Veterinary Record, 2003. **153**(7): p. 202-208.

48. Kittelberger, R., et al., *Atypical scrapie/Nor98 in a sheep from New Zealand.* J Vet Diagn Invest, 2010. **22**(6): p. 863-75.

49. Williams, E.S. and S. Young, *Chronic wasting disease of captive mule deer: a spongiform encephalopathy.* J Wildl Dis, 1980. **16**(1): p. 89-98.

50. Miller, M.W. and E.S. Williams, *Prion disease: Horizontal prion transmission in mule deer.* Nature, 2003. **425**(6953): p. 35-36.

51. Mathiason, C.K., et al., *Infectious Prions in the Saliva and Blood of Deer with Chronic Wasting Disease.* Science, 2006. **314**(5796): p. 133-136.

52. Safar, J.G., et al., *Transmission and detection of prions in feces.* J Infect Dis, 2008. **198**(1): p. 81-9.

53. Sigurdson, C.J., *A prion disease of cervids: chronic wasting disease.* Vet Res, 2008. **39**(4): p. 41.

54. Kong, Q., et al., *Chronic wasting disease of elk: transmissibility to humans examined by transgenic mouse models.* J Neurosci, 2005. **25**(35): p. 7944-9.

55. Tamgüney, G., et al., *Transmission of elk and deer prions to transgenic mice.* J Virol, 2006. **80**(18): p. 9104-9114.

56. Raymond, G.J., et al., *Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease.* Embo j, 2000. **19**(17): p. 4425-30.

57. Samantha, M., et al., *Human Prion Disease and Relative Risk Associated with Chronic Wasting Disease.* Emerging Infectious Diseases, 2006. **12**(10): p. 1527-1535.

58. Wells, G.A., et al., *A novel progressive spongiform encephalopathy in cattle.* Veterinary Record, 1987. **121**(18): p. 419-420.

59. Hope, J., et al., *Fibrils from brains of cows with new cattle disease contain scrapie-associated protein.* Nature, 1988. **336**(6197): p. 390-2.

60. Wilesmith, J.W., et al., *Bovine spongiform encephalopathy: epidemiological features 1985 to 1990.* Vet Rec, 1992. **130**(5): p. 90-4.

61. Bons, N., et al., *Natural and experimental oral infection of nonhuman primates by bovine spongiform encephalopathy agents.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 4046-4051.

62. Hill, A.F., et al., *The same prion strain causes vCJD and BSE.* Nature, 1997. **389**(6650): p. 448-450.

63. Glasse, R., *Cannibalism in the Kuru region of New Guinea.* Trans N Y Acad Sci, 1967. **29**(6): p. 748-54.

64. Bernoulli, C., et al., *Danger of accidental person-to-person transmission of Creutzfeldt-Jakob disease by surgery.* Lancet, 1977. **1**(8009): p. 478-9.

65. Brown, P., et al., *Potential epidemic of Creutzfeldt-Jakob disease from human growth hormone therapy.* N Engl J Med, 1985. **313**(12): p. 728-31.

66. Buchanan, C.R., M.A. Preece, and R.D. Milner, *Mortality, neoplasia, and Creutzfeldt-Jakob disease in patients treated with human pituitary growth hormone in the United Kingdom.* Bmj, 1991. **302**(6780): p. 824-8.

67. Fradkin, J.E., et al., *Creutzfeldt-Jakob disease in pituitary growth hormone recipients in the United States.* Jama, 1991. **265**(7): p. 880-4.

68. Healy, D.L. and J. Evans, *Creutzfeldt-Jakob disease after pituitary gonadotrophins.* Bmj, 1993. **307**(6903): p. 517-8.

69. Kitamoto, T., R. Iizuka, and J. Tateishi, *An amber mutation of prion protein in Gerstmann-Straussler syndrome with mutant PrP plaques.* Biochem Biophys Res Commun, 1993. **192**(2): p. 525-31.

70. Ghetti, B., et al., *Vascular variant of prion protein cerebral amyloidosis with tau-positive neurofibrillary tangles: the phenotype of the stop codon 145 mutation in PRNP.* Proc Natl Acad Sci U S A, 1996. **93**(2): p. 744-748.

71. Hainfellner, J.A., et al., *The original Gerstmann-Straussler-Scheinker family of Austria: divergent clinicopathological phenotypes but constant PrP genotype.* Brain Pathol, 1995. **5**(3): p. 201-11.

72. Gambetti, P., et al., *Fatal familial insomnia and familial Creutzfeldt-Jakob disease: clinical, pathological and molecular features.* Brain Pathol, 1995. **5**(1): p. 43-51.

73. Palmer, M.S., et al., *Homozygous prion protein genotype predisposes to sporadic Creutzfeldt-Jakob disease.* Nature, 1991. **352**(6333): p. 340-2.

74. Maignien, T., et al., *Pathogenesis of the oral route of infection of mice with scrapie and bovine spongiform encephalopathy agents.* Journal of General Virology, 1999. **80**(11): p. 3035-3042.

75. Jeffrey, M., et al., *Oral inoculation of sheep with the agent of bovine spongiform encephalopathy (BSE). 1. Onset and distribution of disease-specific PrP accumulation in brain and viscera.* J Comp Pathol, 2001. **124**(4): p. 280-9.

76. Sigurdson, C.J., et al., *Oral transmission and early lymphoid tropism of chronic wasting disease PrPres in mule deer fawns (Odocoileus hemionus).* J Gen Virol, 1999. **80**(10): p. 2757-2764.

77. Mitchell, G.B., et al., *Experimental oral transmission of chronic wasting disease to reindeer (Rangifer tarandus tarandus).* PLoS One, 2012. **7**(6): p. e39055.

78. Heppner, F.L., et al., *Transepithelial prion transport by M cells.* Nat Med, 2001. **7**(9): p. 976-7.

79. Donaldson, D.S., et al., *M cell-depletion blocks oral prion disease pathogenesis.* Mucosal Immunol, 2012. **5**(2): p. 216-225.

80. Huang, F.-P., et al., *Migrating intestinal dendritic cells transport PrPSc from the gut.* J Gen Virol, 2002. **83**(1): p. 267-271.

81. Luhr, K.M., et al., *Processing and degradation of exogenous prion protein by CD11c(+) myeloid dendritic cells in vitro.* J Virol, 2002. **76**(23): p. 12259-64.

82. Luhr, K.M., et al., *Scrapie protein degradation by cysteine proteases in CD11c+ dendritic cells and GT1-1 neuronal cells.* J Virol, 2004. **78**(9): p. 4776-82.

83. Raymond, C.R., P. Aucouturier, and N.A. Mabbott, *In Vivo Depletion of CD11c+ Cells Impairs Scrapie Agent Neuroinvasion from the Intestine.* J Immunol, 2007. **179**(11): p. 7758-7766.

84. Kitamoto, T., et al., *Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease.* J Virol, 1991. **65**(11): p. 6292-5.

85. van Keulen, L.J., et al., *Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie.* J Clin Microbiol, 1996. **34**(5): p. 1228-31.

86. Brown, K.L., et al., *Scrapie replication in lymphoid tissues depends on prion protein-expressing follicular dendritic cells.* Nat Med, 1999. **5**(11): p. 1308-1312.

87. Ford, M.J., et al., *Selective expression of prion protein in peripheral tissues of the adult mouse.* Neuroscience, 2002. **113**(1): p. 177-92.

88. Jeffrey, M., et al., *Sites of prion protein accumulation in scrapie-infected mouse spleen revealed by immuno-electron microscopy.* J Pathol, 2000. **191**(3): p. 323-32.

89. Mabbott, N.A., et al., *Temporary depletion of complement component C3 or genetic deficiency of C1q significantly delays onset of scrapie.* Nat Med, 2001. **7**(4): p. 485-487.

90. Klein, M.A., et al., *Complement facilitates early prion pathogenesis.* Nat Med, 2001. **7**(4): p. 488-492.

91. Zabel, M.D., et al., *Stromal Complement Receptor CD21/35 Facilitates Lymphoid Prion Colonization and Pathogenesis.* J Immunol, 2007. **179**(9): p. 6144-6152.

92. Klein, M.A., et al., *PrP expression in B lymphocytes is not required for prion neuroinvasion.* Nat Med, 1998. **4**(12): p. 1429-33.

93. Montrasio, F., et al., *B lymphocyte-restricted expression of prion protein does not enable prion replication in prion protein knockout mice.* Proc Natl Acad Sci U S A, 2001. **98**(7): p. 4034-7.

94. Mabbott, N.A., et al., *Tumor necrosis factor alpha-deficient, but not interleukin-6-deficient, mice resist peripheral infection with scrapie.* J Virol, 2000. **74**(7): p. 3338-44.

95. Prinz, M., et al., *Lymph nodal prion replication and neuroinvasion in mice devoid of follicular dendritic cells.* Proc Natl Acad Sci U S A, 2002. **99**(2): p. 919-24.

96. Mabbott, N.A., et al., *Temporary blockade of the tumor necrosis factor receptor signaling pathway impedes the spread of scrapie to the brain.* J Virol, 2002. **76**(10): p. 5131-9.

97. Montrasio, F., et al., *Impaired Prion Replication in Spleens of Mice Lacking Functional Follicular Dendritic Cells.* Science, 2000. **288**(5469): p. 1257-1259.

98. Mabbott, N.A., et al., *Follicular dendritic cell dedifferentiation by treatment with an inhibitor of the lymphotoxin pathway dramatically reduces scrapie susceptibility.* J Virol, 2003. **77**(12): p. 6845-54.

99. Mohan, J., M.E. Bruce, and N.A. Mabbott, *Follicular dendritic cell dedifferentiation reduces scrapie susceptibility following inoculation via the skin.* Immunology, 2005. **114**(2): p. 225-34.

100. Mabbott, N.A., et al., *Temporary inactivation of follicular dendritic cells delays neuroinvasion of scrapie.* Nat Med, 2000. **6**(7): p. 719-720.

101. Aguzzi, A. and C.J. Sigurdson, *Antiprion immunotherapy: to suppress or to stimulate?* Nat Rev Immunol, 2004. **4**(9): p. 725-736.

102. Beekes, M. and P.A. McBride, *Early accumulation of pathological PrP in the enteric nervous system and gut-associated lymphoid tissue of hamsters orally infected with scrapie.* Neurosci Lett, 2000. **278**(3): p. 181-4.

103. Hoffmann, C., et al., *Prions spread via the autonomic nervous system from the gut to the central nervous system in cattle incubating bovine spongiform encephalopathy.* J Gen Virol, 2007. **88**(3): p. 1048-1055.

104. Haik, S., et al., *The sympathetic nervous system is involved in variant Creutzfeldt-Jakob disease.* Nat Med, 2003. **9**(9): p. 1121-3.

105. McBride, P.A. and M. Beekes, *Pathological PrP is abundant in sympathetic and sensory ganglia of hamsters fed with scrapie.* Neurosci Lett, 1999. **265**(2): p. 135-8.

106. Sigurdson, C.J., et al., *PrP(CWD) in the myenteric plexus, vagosympathetic trunk and endocrine glands of deer with chronic wasting disease.* J Gen Virol, 2001. **82**(Pt 10): p. 2327-34.

107. Heggebo, R., et al., *Disease-associated PrP in the enteric nervous system of scrapie-affected Suffolk sheep.* J Gen Virol, 2003. **84**(Pt 5): p. 1327-38.

108. Glatzel, M., et al., *Sympathetic innervation of lymphoreticular organs is rate limiting for prion neuroinvasion.* Neuron, 2001. **31**(1): p. 25-34.

109. Fevrier, B., et al., *Cells release prions in association with exosomes.* Proc Natl Acad Sci U S A, 2004. **101**(26): p. 9683-9688.

110. Gousset, K., et al., *Prions hijack tunnelling nanotubes for intercellular spread.* Nat Cell Biol, 2009. **11**(3): p. 328-336.

111. Silveira, J.R., et al., *The most infectious prion protein particles.* Nature, 2005. **437**(7056): p. 257-261.

112. Gavier-Widén, D., et al., *Diagnosis of Transmissible Spongiform Encephalopathies in Animals: A Review.* J Vet Diagn Invest, 2005. **17**(6): p. 509-527.

113. Prusiner, S.B., et al., *Measurement of the scrapie agent using an incubation time interval assay.* Ann Neurol, 1982. **11**(4): p. 353-8.

114. Klöhn, P.C., et al., *A quantitative, highly sensitive cell-based infectivity assay for mouse scrapie prions.* Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11666-11671.

115. Saborio, G.P., B. Permanne, and C. Soto, *Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding.* Nature, 2001. **411**(6839): p. 810-813.

116. Saá, P., J. Castilla, and C. Soto, *Ultra-efficient Replication of Infectious Prions by Automated Protein Misfolding Cyclic Amplification.* J Biol Chem, 2006. **281**(46): p. 35245-35252.

117. Atarashi, R., et al., *Ultrasensitive detection of scrapie prion protein using seeded conversion of recombinant prion protein.* Nat Methods, 2007. **4**(8): p. 645-50.

118. Saá, P., J. Castilla, and C. Soto, *Presymptomatic Detection of Prions in Blood.* Science, 2006. **313**(5783): p. 92-94.

119. Haley, N.J., et al., *Detection of sub-clinical CWD infection in conventional test-negative deer long after oral exposure to urine and feces from CWD+ deer.* PLoS One, 2009. **4**(11): p. e7990.

120. Terry, L.A., et al., *Detection of PrPsc in Blood from Sheep Infected with the Scrapie and Bovine Spongiform Encephalopathy Agents.* J Virol, 2009. **83**(23): p. 12552-12558.

121. Maddison, B.C., et al., *Prions Are Secreted in Milk from Clinically Normal Scrapie-Exposed Sheep.* J Virol, 2009. **83**(16): p. 8293-8296.

122. Chen, B., et al., *Estimating prion concentration in fluids and tissues by quantitative PMCA.* Nat Methods, 2010. **7**(7): p. 519-20.

123. Chang, B., et al., *PrP Antibody Binding-Induced Epitope Modulation Evokes Immunocooperativity.* J Neuroimmunol, 2008. **205**(1-2): p. 94-100.

124. Chang, B., et al., *Surround optical fiber immunoassay (SOFIA): An ultra-sensitive assay for prion protein detection.* J Virol Methods, 2009. **159**(1): p. 15-22.

125. Rubenstein, R., et al., *A novel method for preclinical detection of PrPSc in blood.* J Gen Virol, 2010. **91**(7): p. 1883-1892.

126. Atarashi, R., et al., *Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking.* Nat Meth, 2008. **5**(3): p. 211-212.

127. Orru, C.D., et al., *Human variant Creutzfeldt-Jakob disease and sheep scrapie PrP(res) detection using seeded conversion of recombinant prion protein.* Protein Eng Des Sel, 2009. **22**(8): p. 515-21.

128. Rogers, D.R., *Screening for amyloid with the thioflavin-t fluorescent method.* American journal of clinical pathology, 1965. **44**: p. 59-61.

129. Colby, D.W., et al., *Prion detection by an amyloid seeding assay.* Proc Natl Acad Sci U S A, 2007. **104**(52): p. 20914-9.

130. Wilham, J.M., et al., *Rapid end-point quantitation of prion seeding activity with sensitivity comparable to bioassays.* PLoS Pathog, 2010. **6**(12): p. e1001217.

131. Atarashi, R., et al., *Ultrasensitive human prion detection in cerebrospinal fluid by real-time quaking-induced conversion.* Nat Med, 2011. **17**(2): p. 175-8.

132. Bessen, R.A., et al., *Accelerated shedding of prions following damage to the olfactory epithelium.* J Virol, 2012. **86**(3): p. 1777-88.

133. Korth, C., et al., *Prion (PrPSc)-specific epitope defined by a monoclonal antibody.* Nature, 1997. **390**(6655): p. 74-7.

134. Orru, C.D., et al., *Prion disease blood test using immunoprecipitation and improved quaking-induced conversion.* MBio, 2011. **2**(3): p. e00078-11.

135. Zobeley, E., et al., *Infectivity of scrapie prions bound to a stainless steel surface.* Mol Med, 1999. **5**(4): p. 240-3.

136. Luhr, K.M., et al., *Prion adsorption to stainless steel is promoted by nickel and molybdenum.* J Gen Virol, 2009. **90**(Pt 11): p. 2821-8.

137. Edgeworth, J.A., et al., *Detection of prion infection in variant Creutzfeldt-Jakob disease: a blood-based assay.* Lancet, 2011. **377**(9764): p. 487-93.

138. Jackson, G.S., et al., *A highly specific blood test for vCJD.* Blood, 2014. **123**(3): p. 452-3.

CHAPTER 2

DEVELOPMENT OF A RADIOIMMUNOASSAY FOR SCREENING OF PRION DISEASES

2.1 Abstract

The main challenge in the diagnosis of prion disease in animals is the lack of a reliable preclinical assay. Current assays using plasma samples through amplification or extraction of proteinase K-resistant prion protein (PrP^{SC}) face a problem of high false-results rate, because of the low concentrations of PrP^{SC} in plasma samples. In this report, we described the development of radioimmunoassay (RIA) of cellular prion protein (PrP^{C}) by using ^{125}I-labeled residue 143-153 of prion protein (^{125}I-$PrP_{143-153}$). This assay could detect prion protein (PrP) fragments in brain homogenate and plasma sample from healthy sheep. Using RIA, the concentration of $PrP_{143-153}$ was determined as 0.29 μg/g total proteins in a brain homogenate, and 99 pg/ml in a plasma sample. The detection limit of this assay was 31 pg/ml, and the intra- and inter-assay variabilities were <10%. This assay may have a potential to be used for diagnosis of scrapie in sheep and chronic wasting disease (CWD) in cervids. The animals with prion disease are expected to have lower plasma concentrations of $PrP_{143-153}$ than normal animals. This hypothesis will be tested in future studies.

2.2 Introduction

Prion disease is a transmissible neurodegenerative disease with slow progression. Misfolded prion proteins (PrP^{SC}) are regarded as infectious agents and biomarker of prion disease. PrP^{SC} replicates in the cells with high concentration of cellular prion protein (PrP^{C}), such as neuronal cells, and forms aggregates, known as amyloid. The disease with aggregates of PrP^{SC} causes the death of neurons and forms tiny holes in cerebral cortex. Thus, prion disease is also known as transmissible spongiform encephalopathy (TSE). Prion disease has been found in several species, including scrapie in sheep and goats, Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in cervids.

In 1947, the first case of scrapie was found in the United States, and most of scrapie cases are only transmitted within the flock. Although there is no large outbreak of scrapie in the United States, sporadic reports of scrapie indicate loss of export sales which cost producers $20 million per year [1]. In order to eradicate scrapie in the United States, it requires reliable assays for large-scale surveillance. CWD is found in several geographic areas of the United States, especially northeastern Colorado and southeastern Wyoming. A previous study showed Suffolk sheep developed CWD after receiving intracerebral inoculation of brain homogenate from CWD-infected mule deer [2]. Thus, it is still a concern that CWD could be transmitted to other species, especially sheep.

PrP^{SC} is detected in milk, feces and saliva from scrapie-infected sheep [3-5], and it is found in saliva, blood and feces from CWD-infected cervids [6-8]. These

body fluids and excreta could be used for diagnosis of prion disease. In recent years, the low amount of PrP^{SC} in plasma samples was detected by following methods. First, PrP^{SC} is amplified by protein misfolding cyclic amplification (PMCA) and detected by the surround optical fiber immunoassay (SOFIA) [9, 10]. Second, PrP^{SC} in plasma is extracted by PrP^{SC}-specific antibody, and then purified PrP^{SC} is amplified and detected by real time quaking-induced conversion (RT-QuIC) [11, 12]. However, the process of amplifying PrP^{SC} results in a high false positive rate and the assay procedures are complicated. Third, PrP^{SC} in plasma is extracted by stainless steel and the concentration of purified PrP^{SC} is measured by ELISA [13, 14]. However, the false negative rate in this assay is 28.6% [13].

Shed PrP^{C} and PrP^{C} fragments in extracellular fluid are released from cells surface through the cleavage of glycosylphosphatidylinositol (GPI)-anchor on PrP^{C} by a-disintegrin-and-metalloproteinase 10 (ADAM10) [15, 16].

The objective of the present study was to develop a radioimmunoassay (RIA) to detect the plasma concentrations of PrP^{C} in both normal animals and animals infected with scrapie or CWD. In future studies, we will determine whether there is a difference between normal and diseased animals with regard to plasma concentrations of the prion protein.

2.3 Materials and Methods

Materials

Chemicals and their sources are stated as follows: synthetic human residue 143-153 (human $PrP_{143-153}$), SDYEDRYYREN and synthetic ovine residue 143-153

(ovine $PrP_{143-153}$), NDYEDRYYREN (Protein Facility, Iowa State University, IA); ovine recombinant prion protein (recPrP) (Abcam, Cambridge, MA); $Na^{125}I$ and Packard Cobra γ counter 5002 (PerkinElmer, Boston, MA); goat anti-$PrP_{143-153}$ antibody (Aviva Systems Biology, San Diego, CA & GeneTex, Irvine, CA); 6H4 monoclonal antibody (mAb) (Prionics, Switzerland); POM1 mAb (Prionatis AG, Switzerland); chloramine-T, n-octyl-glucoside, 2-(N-morpholino)ethanesulfonic acid (MES), and phenylmethylsulfonylfluoride (PMSF) (Acros Organics, Belgium); G-25 Sephadex, sodium metabisulfite (Sigma-Aldrich, St. Louis, MO); BCA protein assay (Pierce, Rockford, IL); polyethylene glycol (PEG) 8000 (Fisher Scientific, Pittsburg, PA).

Iodination and purification of recPrP and $PrP_{143-153}$

Iodination and purification of recPrP

Five μg of ovine recPrP was dissolved in 30 μl 0.1 M phosphate buffer (pH 7.1) with 0.1% triton X-100, and 1 mCi $Na^{125}I$ was added. Iodination reaction was initiated by adding 10 μl of 0.4 mg/ml chloramine-T. After 4 min, 20 μl of 0.2 mg/ml sodium metabisulfite was added to stop the reaction. The iodinated mixture was loaded into a G-25 Sephadex column, and eluted by 0.1 M phosphate buffer (pH 7.1) with 0.1% triton X-100 at room temperature. One-ml fractions were collected, and 3 μl aliquots were counted by Packard Cobra γ counter 5002. The ^{125}I-recPrP appeared in fraction 4, which was stored at 4°C.

Iodination and purification of $PrP_{143-153}$

Two μg of synthetic human $PrP_{143-153}$ was dissolved in 10 μl of 0.5 M phosphate buffer (pH 7.5). One mCi $Na^{125}I$ was added to solution, and iodination reaction was

initiated by adding 10 μl of 1 mg/ml chloramine-T. After 1 min of reaction, 100 μl of 100 mg/ml bovine serum albumin (BSA) in 0.1 M acetic acid was added to quench surplus ^{125}I. The iodinated mixture was loaded into a G-25 Sephadex column, and column was eluted by 0.1 M acetic acid with 0.1% BSA at 4°C. One-ml fractions were collected, and ^{125}I-$PrP_{143-153}$ appeared in fractions 15-20. The ^{125}I-$PrP_{143-153}$ was stored at 4 ℃.

Homogenization of ovine brain tissue and collection of ovine plasma

Ovine brain tissues (10% w/v) were homogenized by Tissumizer SDT-1810 (Tekmar, Cincinnati, OH) on ice in 25 mM MES (pH 6.5), 150 mM NaCl, 1% triton X-100, 60 mM n-octyl-glucoside, 10 mM PMSF and 1mM EDTA. To remove debris, the homogenate was centrifuged at 3090 X *g* for 30 min at 4°C. Supernatants were collected and stored at -20°C. The protein concentration in brain homogenate was determined by BCA protein assay. Ovine blood samples were collected with 0.2% EDTA and placed on ice. Blood samples were centrifuged at 1373 X *g* for 20 min at 4°C. Plasma was collected and stored at -20°C.

Radioimmunoassay

Radioimmunoassay of recPrP

Polyethylene tubes were used for the assay. One hundred μl of recPrP solution was incubated with 1:10000 POM1 mAb in 0.1 M phosphate buffer (pH 7.5), 0.2% BSA, 0.1% NaN_3 at 4°C for overnight. Then, 100 μl of 125I-recPrP (30,000 cpm) was added to tubes and incubated for overnight. Next day, 1:40 anti-mouse antibody and 1:80 mouse serum were added to each assay tube, and tubes were incubated for another 30 min at 4°C. One hundred μl of horse serum and 1 ml 12% PEG 8000 in

0.85% NaCl were added before centrifugation at 3090 X *g* for 30 min at 4°C. Supernatants were removed, and pellets were counted by Packard Cobra γ counter 5002.

Radioimmunoassay of $PrP_{143-153}$

One hundred µl of ovine brain homogenate or ovine plasma was incubated with 100 µl of 1:4000 goat anti-$PrP_{143-153}$ antibody (0.5 mg/ml stock) in 0.1 M phosphate buffer (pH7.5), 0.2% BSA, 0.1% NaN_3 and 2 mM EDTA at 4°C for overnight. Then, 100 µl of ^{125}I-$PrP_{143-153}$ (30,000 cpm) was added and incubated for overnight. On the third day, 100 µl of 1:20 donkey anti-goat antibody and 100 µl of 1:40 ovine plasma were added to each assay tube, and tubes were incubated for 30 min at 4°C. One hundred µl of horse serum and 1 ml of 12% PEG 8000 in 0.85% NaCl were added to each assay tube immediately before centrifugation at 3090 X *g* for 30 min at 4°C. In the assay of ovine plasma, on the third day, 100 µl of 1:20 donkey anti-goat antibody and 100 µl of ovine plasma were added and tubes were incubated for 5 min at 4°C. Then, 1 ml of 12% PEG 8000 in 0.85% NaCl was added and centrifuged at 3090 X *g* for 30 min at 4°C. Supernatants were removed and pellets were counted by Packard Cobra γ counter 5002. The standard curve was set up using synthetic $PrP_{143-153}$.

Digestion of prion protein with proteinase K

Ovine brain homogenates were incubated with 0.6, 1.9, 5.6 µg/ml proteinase K in 50 mM Tris buffer (pH 8.0) and 10 mM $CaCl_2$ at 60°C for 1 hr. The reactions were terminated by adding 10 mM PMSF in isopropanol, and samples were stored at 4°C.

Data Analysis

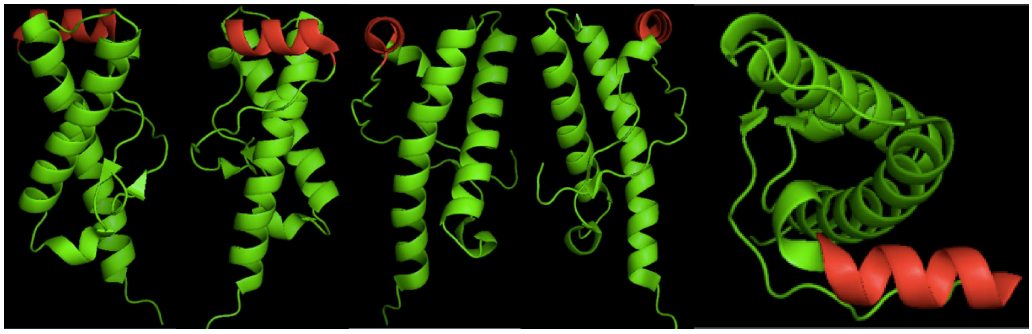
% Bound was calculated by cpm in the pellet over the total cpm. The standard curve was composed of X: $\ln(concentration\ of\ PrP_{143-153})$ and Y: $ln\left(\frac{\frac{Measured\ count-blank}{Count\ of\ zero-blank}}{1-\frac{Measured\ count-blank}{Count\ of\ zero-blank}}\right)$, and it was fit by the linear regression.

2.4 Results

We attempted to establish the RIA of recPrP in the early stage of the research; we intended to label recPrP with ^{125}I and purify the ^{125}I-recPrP. ^{125}I-recPrP interacted with POM1 mAb, and the displacement by recPrP was detected. However, it was not practical to use ^{125}I-recPrP as a tracer in RIA due to two reasons: First, the specific binding was 14.9%, but the non-specific binding was 34.3%. Second, the life span of the tracer was only a week. These two problems might be caused by the aggregation of recPrP. To avoid the problem of aggregation, we looked for a region in PrP instead of the full-length of PrP as the tracer candidate. We prepared $PrP_{143\text{-}153}$ as the tracer in RIA because of following four reasons: First, the internal region 143-153 is the first α-helix in the globular structure of PrP, and this structure is not imbedded in the globular structure (Figure 1A-D). Second, this sequence is highly conserved in different species (Figure 1F). In human and cervid/ovine $PrP_{143\text{-}153}$, there is only one amino acid difference in the sequence. Thus, the RIA of $PrP_{143\text{-}153}$ was developed to assay the samples from different species. Third, there are three commercial antibodies against this region. 6H4 mAb is against residue 144-152 of human PrP (DYEDRYYRE) [17]. The pair-wise mapping study indicated that POM1 mAb shares the similar epitope with 6H4 mAb [18]. The goat

anti-$PrP_{143-153}$ antibody used in this study was raised in a goat against synthetic $PrP_{143-153}$ (SDYEDRYYREN). Fourth, tyrosine is required for iodination of peptides, and there are three molecules of tyrosine in this region.

(A) (B) (C) (D) (E)



(F)

Human:	S	D	Y	D	R	Y	Y	R	N
Bovine:	.	.	.	.	.	.	.	.	.
Pig:	.	.	.	.	.	.	.	.	.
Deer:	N	.	.	.	.	.	.	.	.
Ovine:	N	.	.	.	.	.	.	.	.
Mouse:	N	.	W	.	.	.	.	.	.
Hamster:	N	.	W	.	.	.	.	.	.

Figure 1. (A-E) The globular structure of ovine prion protein. The red region is the first α-helix structure with the sequence of SDYEDRYYREN. (F) The residues of $PrP_{145-153}$ in different species; the sequence of human PrP is based on NCBI protein database, NP_000302.1.

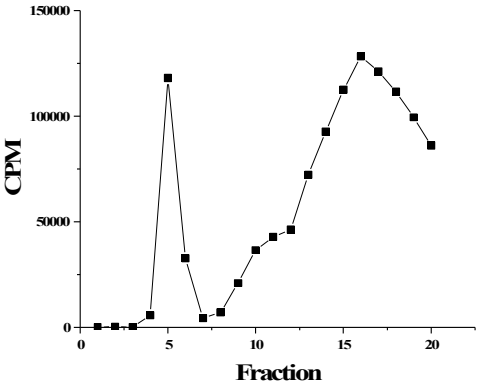
The synthetic human $PrP_{143-153}$ was iodinated by ^{125}I, and surplus ^{125}I was quenched by BSA (100 mg/ml) that prevented it from reacting with $PrP_{143-153}$. Through antibody binding test, it was found that fraction 5 contained BSA, fractions

11-12 contained free ^{125}I and fractions 15-20 contained ^{125}I-PrP$_{143-153}$ (Figure 2A). Fraction 16 had the highest binding by anti-PrP$_{143-153}$ antibody. Unexpectedly, ^{125}I-PrP$_{143-153}$ did not interact with either 6H4 mAb or POM1 mAb (data not shown). Because of the difference in first N-terminal amino acid between human and ovine PrP$_{143-153}$, it might cause a different interaction between PrP$_{143-153}$ and anti-PrP$_{143-153}$ antibody. In order to understand whether anti-PrP$_{143-153}$ antibody could distinguish the difference, we used both human PrP$_{143-153}$ and ovine PrP$_{143-153}$ to set standard curves in this RIA (Figure 2B). The result showed there was no difference between two standard curves, which indicated that this RIA does not distinguish human PrP$_{143-153}$ and ovine PrP$_{143-153}$.

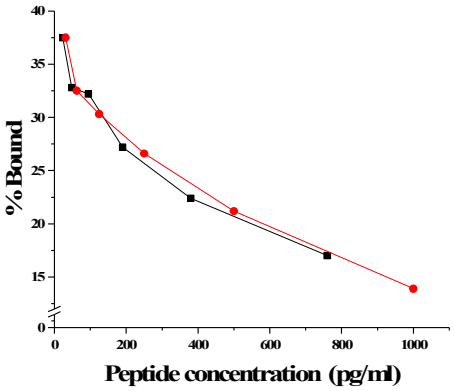
Ovine recPrP was used to compete with the tracer, but there was no competition. In other words, recPrP was not detected by the RIA of PrP$_{143-153}$, and thus this RIA could not be used to detect recPrP. However, the serial dilution of ovine brain homogenate showed the parallelism with the standard curve of PrP$_{143-153}$ in the antibody binding activity (Figure 2C). Also, the standard curve of PrP$_{143-153}$ showed that detection limit of the RIA was 31 pg/ml and R^2 was >0.99. The RIA did not detect recPrP, but "something" was detected in the ovine brain homogenate by this assay. Because the molecular weight of full-length PrP is 23 times larger than ^{125}I-PrP$_{143-153}$, full-length PrP might be difficult to compete with the tracer. Our results suggested that the brain homogenate contains not only intact PrP but also the fragments of PrP^C that can compete with the tracer on the antibody against PrP$_{143-153}$. We speculate that "something" is the fragments of PrP^C.

The RIA was validated by the homogenate of ovine brain (Table 1). The inter-assay variability was 7%, and the recovery was 105%. The intra-assay variability was 7-10%, and the PrP^C fragments in ovine brain samples was 0.29 μg/g total proteins.

(A)



(B)



(C)

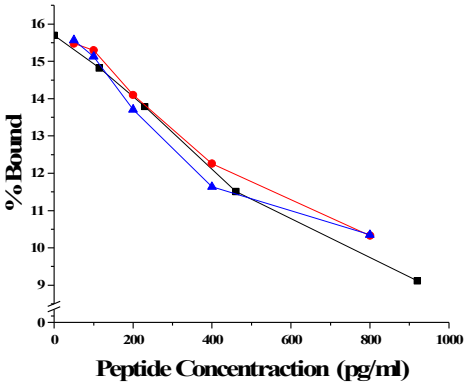


Figure 2. Purification of ^{125}I-PrP$_{143-153}$ and parallelism between the standard curve and serial dilutions of ovine brain samples. (A) Iodinated mixture was purified by Sephadex G-25 column, and 3 µl aliquots were counted. (B) Standards of 48 to 760 pg/ml human PrP$_{143-153}$ (■) and standards of 63 to 1000 pg/ml ovine PrP$_{143-153}$ (●). (C) 1:1 serial dilution of ovine brain homogenates yield binding results that paralleled with those of the standard curve of PrP$_{143-153}$. Standards of 115 to 920 pg/ml PrP$_{143-153}$ were used (■), two independent curves of ovine brain homogenates diluted from 1:5 to 1:80, and the curves shifted horizontally (●) (▲). Each value represents the data from duplicates of an independent RIA.

Table 1. 920 pg/ml PrP$_{143-153}$ was used as a reference standard that was repeated 5 times to calculate inter-assay variability and recovery. Samples were ovine brain homogenate being diluted 1:8, and each sample was assayed 4 times to calculate intra-assay variability and mean concentration of PrP^{C} fragment in an ovine brain homogenate. Each value represents the repeat of the reference standard samples from duplicates of the RIA. Coefficient of variation (CV) = standard deviation/mean.

Validation of the RIA

Inter-assay Variation

Reference (920 pg/ml)					Average	Recovery	CV
969.6	1023.8	843.7	988.2	987.1	962.5	104.62%	7.19%

Intra-assay Variation

Sample				Average	CV
286.3	251.1	308.9	273.6	280.0	8.64%
383.7	345.1	385.5	413.2	381.9	7.32%
280.6	254.8	285.7	326.2	286.8	10.3%
509.8	520.4	500.8	579.4	527.6	6.72%

In order to confirm the hypothesis that this RIA could only detect PrP^C fragments containing $PrP_{143-153}$ instead of intact PrP^C, the brain homogenate (9.1 mg protein/ml) was pretreated with proteinase K that is widely used to digest PrP^C. The ovine brain homogenates were digested by 0.6, 1.9 and 5.6 μg/ml proteinase K at 60°C for 1 hr, which showed lower % bound than control group in the RIA (Figure 3A). These results demonstrated that the digestion by proteinase K released the fragments from intact PrP^C, and these fragments were able to compete with the tracer for the antibody binding. Moreover, the amount of proteinase K-released fragments was proportional to the dose of proteinase K. The dose-dependency of proteinase K further supported the hypothesis that only fragments containing $PrP_{143-153}$ could be detected in this RIA.

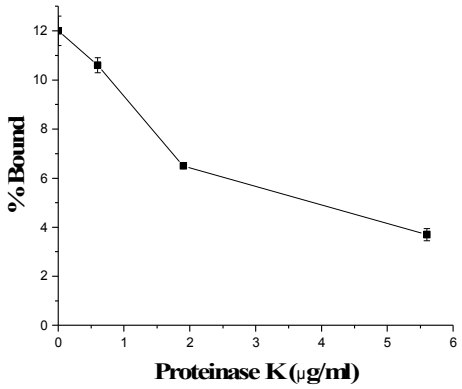


Figure 3. Pretreatment of ovine brain homogenate with proteinase K releases PrP^C fragments from intact protein. Ovine brain homogenate was treated with 0.6, 1.9 and 5.6 μg/ml proteinase K for 1hr at 60°C, and reaction was terminated by 10 mM PMSF.

Through RIA, the concentration of fragments containing $PrP_{143-153}$ in ovine plasma samples was detected as 99 ± 9.7 pg/ml. In order to know whether other components in plasma distorted the RIA, we used standard addition method to test. The plasma samples were spiked with 24, 48, 95, 190 and 380 pg/ml analyte ($PrP_{143-153}$). The curve of spiked plasma samples paralleled with the standard curve of $PrP_{143-153}$, which indicated that there were no interferents in plasma samples to affect the RIA (Figure 4A). In addition, the concentration of PrP^C fragments in plasma was equal to the difference between observed concentration and analyte concentration. The mean of differences was 118 pg/ml (Table 2). This value was similar to that of unspiked ovine plasma sample (99 pg/ml), which indicated the concentration of PrP^C fragments in the plasma sample was ~108 pg/ml. Based on the results of measurement of unspiked samples and standard addition method, we concluded that the RIA could detect the PrP^C fragments containing $PrP_{143-153}$ in plasma samples.

In order to explore how fast peptides are degraded in plasma samples with 0.2% EDTA, we added the 920 pg/ml $PrP_{143-153}$ standard to ovine plasma and incubated it at 37°C. Samples were collected at each time point and stored at 4°C until assay. Comparing with 0 min, the concentration of $PrP_{143-153}$ was 97.7% at 3 hr incubation, 92.6% at 6 hr, 85.9% at 12 hr and 77.4% at 24 hr (Figure 4B). This result suggested that $PrP_{143-153}$ is stable in plasma, and thus the plasma samples for the RIA are well-preserved when 0.2% EDTA is used as the anticoagulant.

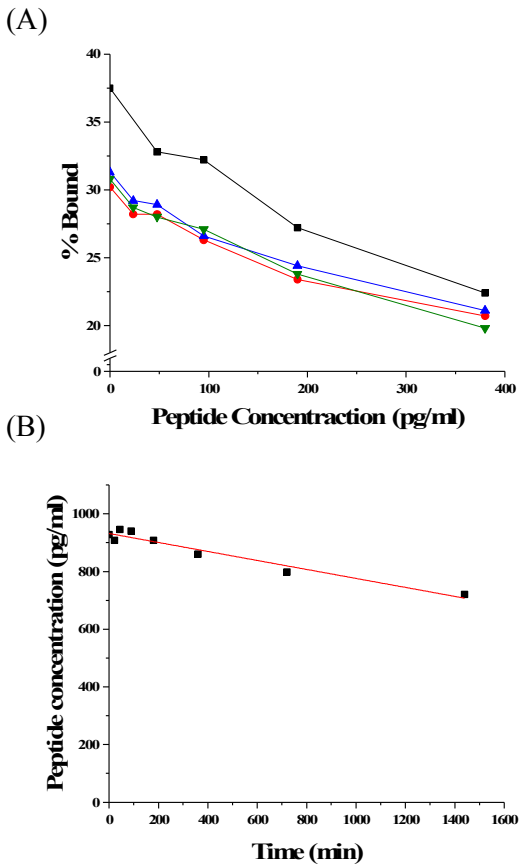


Figure 4. Ovine plasma spiked with $PrP_{143-153}$ and degradation of $PrP_{143-153}$ in ovine plasma. (A) The parallelism between the standard curve of $PrP_{143-153}$ and the serially diluted ovine plasma samples spiked with $PrP_{143-153}$. Standard curve of 24 to 760 pg/ml $PrP_{143-153}$ (■) and three samples of ovine plasma spiked with 24 to 380 pg/ml $PrP_{143-153}$ (●)(▲)(▼). (B) $PrP_{143-153}$ was stable in ovine plasma at 37°C. 920 pg/ml $PrP_{143-153}$ was added to ovine plasma, and tubes were incubated at 37°C for different time points. Each value represents the data from duplicates of the RIA.

Table 2. The concentration of PrP^C fragments in ovine plasma sample = observed - analyte ($PrP_{143-153}$). Each value represents the data from duplicates of the RIA.

Standard addition of ovine plasma sample

Analyte (pg/ml)	Observed (pg/ml) (Mean ± SE)	Observed mean - Analyte
24	148 ± 7.1	124
48	157 ± 7.8	109
95	208 ± 7.7	113
190	314 ± 13	124
380	499 ± 27	119
Mean (Observed - Analyte)		118

2.5 Discussion

Our present findings showed that ^{125}I-recPrP was not appropriate to be used as a tracer in RIA due to high non-specific binding and short life span. In contrast, ^{125}I-$PrP_{143-153}$ as a tracer and goat anti-$PrP_{143-153}$ as first antibody performed well in the RIA. The present study showed the RIA of $PrP_{143-153}$ detected the concentration of PrP^C fragments in brain homogenate and plasma samples from sheep. In healthy sheep, the concentration of PrP^C fragments was 0.29 µg/g total proteins in a brain homogenate, and 99 pg/ml in a plasma sample. The sensitivity of the RIA was 31 pg/ml, and both intra-assay and inter-assay variability was <10%. In addition, <10% of $PrP_{143-153}$ was degrade in plasma after 6 hr of incubation at 37°C.

Is $PrP_{143-153}$ specific for PrP^C? We did a search of the sequence SDYEDRYYREN in the NCBI protein database and RCSB protein data bank. The results indicated that this sequence is only in PrP^C, but not other known proteins. Thus, the concentration of the fragments only reflects the degradation of PrP^C. The data showed fragments containing $PrP_{143-153}$ in ovine brain homogenate was 0.29 µg/g

of total proteins. If the amount of PrP^C fragments is calculated to express that of intact PrP^C (MW of $PrP_{143-153}$: MW of PrP^C = 1,500 : 35,000), it indicates that this amount of fragments would be released from 6.1 μg of intact PrP^C. A previous study showed that the concentration of PrP^C in hamster brain homogenate was 70 μg/g total protein [19]. By taking these two numbers into consideration, we speculate that 8.7% of PrP^C would be constantly degraded and released from the plasma membrane of PrP^C-containing cells, e.g., neurons.

We hypothesize that the RIA of $PrP_{143-153}$ is difficult to detect intact PrP^C in the brain homogenate for three reasons: First, the data showed that 6.1 μg PrP^C was detected in an ovine brain homogenate, which is 10 times less than the result from a previous study. Second, the brain homogenate digested by proteinase K showed more detectable fragments have been released. Third, ovine recPrP was not detected by this RIA. However, unlike PrP^C, recPrP does not have the posttranslational modification. Therefore, it is necessary to use pure PrP^C to further verify the hypothesis that the RIA of $PrP_{143-153}$ is difficult to detect intact PrP^C.

Can goat anti-$PrP_{143-153}$ antibody recognize the peptide whose sequence is not fully identical to human $PrP_{143-153}$? Although the first amino acid of ovine $PrP_{143-153}$ is different from that of human $PrP_{143-153}$, there was no difference between standard curves set by human $PrP_{143-153}$ and ovine $PrP_{143-153}$ (Figure 2B). Also, the serial dilution of ovine brain homogenate showed parallelism with the standard curve of human $PrP_{143-153}$. These results suggested the difference of the first amino acid of N-terminal of the peptide does not affect the interaction between goat anti-$PrP_{143-153}$ antibody and PrP^C fragments. In addition, cervids have an identical $PrP_{143-153}$ with

sheep. Therefore, this RIA can be used to analyze the samples from cervids as well. For future studies, one should use the ovine peptide as standards to assay ovine/cervid plasma samples.

Through epitope mapping by the 13-mer synthetic peptide, a previous study indicated that the epitope of 6H4 mAb recognizes 144-152 in human PrP [17]. However, another study showed that 6H4 mAb did not bind the 25-mer synthetic peptide [18]. In addition, in the pair-wise epitope mapping, POM1 mAb competed with 6H4 mAb, which suggested the epitope of POM1 mAb corresponds to 6H4 epitope. Our results showed that neither 6H4 mAb nor POM1 mAb bound $PrP_{143-153}$. In contrast, there was a binding between $PrP_{143-153}$ and goat anti-$PrP_{143-153}$ antibody. 6H4 mAb was raised using bovine recPrP as the antigen in *Prnp*$^{0/0}$ mice, and POM1 mAb was raised using mouse recPrP in *Prnp*$^{0/0}$ mice. On the contrary, goat anti-$PrP_{143-153}$ antibody was raised using synthetic peptide with the sequence of C-SDYEDRYYREN as an antigen. The discrepancy of these antibodies might be caused by different physical structures of $PrP_{143-153}$ between recPrP and the synthetic peptide. Although 6H4 mAb and POM1 mAb have the major epitope on first α-helix of PrP, the interaction might be assisted by other amino acids or structures near this region.

What is the relationship between the amount of PrP^C and prion disease? In the diseased animals, a large amount of PrP^C is converted to PrP^{SC} and forms aggregates that are resistant to degradation. Eventually, due to the depletion of PrP^C, the production of PrP^C fragments is expected to decrease. Thus, we hypothesize that the amount of PrP^C fragments ($PrP_{143-153}$) in animals with prion disease is lower than

that of healthy animals. Because no sufficient evidence supports that PrP^C can be transported through the blood brain barrier, the PrP^C in the blood most likely comes from peripheral nerve systems (PNS), platelets and follicular dendrite cells (FDCs) [20, 21]. At the early stage of a prion disease, PrP^{SC} is replicated and accumulated on FDCs in secondary lymphoid tissues and then slowly invades PNS. This process might decrease PrP^C fragments in the blood. This hypothesis needs to be tested through assaying the plasma samples from animals with and without prion disease.

In conclusion, we developed the RIA of $PrP_{143-153}$, and it could detect PrP^C fragments in brain homogenate and plasma samples. Based on our hypothesis, this RIA has a potential to be used as a screening test for diagnosis of scrapie and CWD using plasma samples.

2.6 References

1. Kahler, S.C., *The rationale for ridding U.S. of scrapie.* J Am Vet Med Assoc, 2002. **220**(9): p. 1280-1.
2. Hamir, A.N., et al., *Transmission of chronic wasting disease of mule deer to Suffolk sheep following intracerebral inoculation.* J Vet Diagn Invest, 2006. **18**(6): p. 558-65.
3. Terry, L.A., et al., *Detection of prions in the faeces of sheep naturally infected with classical scrapie.* Vet Res, 2011. **42**: p. 65.
4. Tamguney, G., et al., *Salivary prions in sheep and deer.* Prion, 2012. **6**(1): p. 52-61.
5. Konold, T., et al., *Evidence of effective scrapie transmission via colostrum and milk in sheep.* BMC Vet Res, 2013. **9**: p. 99.

6. Miller, M.W. and E.S. Williams, *Prion disease: Horizontal prion transmission in mule deer.* Nature, 2003. **425**(6953): p. 35-36.

7. Mathiason, C.K., et al., *Infectious Prions in the Saliva and Blood of Deer with Chronic Wasting Disease.* Science, 2006. **314**(5796): p. 133-136.

8. Safar, J.G., et al., *Transmission and detection of prions in feces.* J Infect Dis, 2008. **198**(1): p. 81-9.

9. Saborio, G.P., B. Permanne, and C. Soto, *Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding.* Nature, 2001. **411**(6839): p. 810-813.

10. Rubenstein, R., et al., *A novel method for preclinical detection of PrPSc in blood.* J Gen Virol, 2010. **91**(7): p. 1883-1892.

11. Atarashi, R., et al., *Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking.* Nat Meth, 2008. **5**(3): p. 211-212.

12. Orru, C.D., et al., *Prion disease blood test using immunoprecipitation and improved quaking-induced conversion.* MBio, 2011. **2**(3): p. e00078-11.

13. Edgeworth, J.A., et al., *Detection of prion infection in variant Creutzfeldt-Jakob disease: a blood-based assay.* Lancet, 2011. **377**(9764): p. 487-93.

14. Jackson, G.S., et al., *A highly specific blood test for vCJD.* Blood, 2014. **123**(3): p. 452-3.

15. Vincent, B., et al., *The disintegrins ADAM10 and TACE contribute to the constitutive and phorbol ester-regulated normal cleavage of the cellular prion protein.* J Biol Chem, 2001. **276**(41): p. 37743-6.

16. Laffont-Proust, I., et al., *The N-terminal cleavage of cellular prion protein in the human brain.* FEBS Letters, 2005. **579**(28): p. 6333-6337.

17. Korth, C., et al., *Prion (PrPSc)-specific epitope defined by a monoclonal antibody.* Nature, 1997. **390**(6655): p. 74-7.

18. Polymenidou, M., et al., *The POM monoclonals: a comprehensive set of antibodies to non-overlapping prion protein epitopes.* PLoS One, 2008. **3**(12): p. e3872.

19. Pan, K.M., N. Stahl, and S.B. Prusiner, *Purification and properties of the cellular prion protein from Syrian hamster brain.* Protein Sci, 1992. **1**(10): p. 1343-52.

20. Holada, K., et al., *Expression of cellular prion protein on platelets from patients with gray platelet or Hermansky-Pudlak syndrome and the protein's association with alpha-granules.* Haematologica, 2006. **91**(8): p. 1126-9.

21. Brouckova, A. and K. Holada, *Cellular prion protein in blood platelets associates with both lipid rafts and the cytoskeleton.* Thromb Haemost, 2009. **102**(5): p. 966-74.