

## Review

# Susceptibility of cell substrates to PrP<sup>Sc</sup> infection and safety control measures related to biological and biotherapeutic products

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**Abbreviations:** CJD, creutzfeldt-jakob disease; TSE, transmissible spongiform encephalopathies; vCJD, variant form CJD; CWD, chronic wasting disease; BSE, bovine spongiform encephalopathy

**Key words:** prion, transmission, detection, tissue, blood transfusion, biologics, biotherapeutics, vaccine, cell substrates

Concerns over the potential for infectious prion proteins to contaminate human biologics and biotherapeutics have been raised from time to time. Transmission of the pathogenic form of prion protein (PrP<sup>Sc</sup>) through veterinary vaccines has been observed, yet no human case through the use of vaccine products has been reported. However, iatrogenic transmissions of PrP<sup>Sc</sup> in humans through blood components, tissues and growth hormone have been reported. These findings underscore the importance of reliable detection or diagnostic methods to prevent the transmission of prion diseases, given that the number of asymptomatic infected individuals remains unknown, the perceived incubation time for human prion diseases could be decades, and no cure of the diseases has been found yet. A variety of biochemical and molecular methods can selectively concentrate PrP<sup>Sc</sup> to facilitate its detection in tissues and cells. Furthermore, some methods routinely used in the manufacturing process of biological products have been found to be effective in reducing PrP<sup>Sc</sup> from the products. Questions remain unanswered as to the validation criteria of these methods, the minimal infectious dose of the PrP<sup>Sc</sup> required to cause infection and the susceptibility of cells used in gene therapy or the manufacturing process of biological products to PrP<sup>Sc</sup> infections. Here, we discuss some of these challenging issues.

## Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative diseases linked to a single, misfolded prion protein (PrP<sup>Sc</sup>).<sup>91</sup> The misfolded prion proteins are infectious and have been identified in humans and in a variety of animal species. The diseases

caused by these infectious prions include scrapie in sheep and goats, chronic wasting disease (CWD) in deer, elk and moose, bovine spongiform encephalopathy (BSE) in cattle and assorted forms of Creutzfeldt-Jakob disease (CJD) including familial, sporadic, and the recently discovered variant forms (vCJD) in humans.<sup>4,38,35,43-45,88</sup> TSEs are characterized pathologically by the vacuolation and loss of neurons in the central nervous system and the accumulation of the disease isoform (PrP<sup>Sc</sup>) of host normal cellular prion protein (PrP<sup>C</sup>).<sup>65</sup>

PrP<sup>C</sup> has been found in various types of cells and tissues including the central nervous system, myoblasts and myotubes, vascular endothelial cells, reproductive tissues and cells of hemopoietic lineage.<sup>11,72,74,79,85</sup> In animals or humans suffering from TSEs, PrP<sup>Sc</sup> has been identified in numerous tissues including the central and peripheral nervous systems, lymphoid tissues, muscles and mammary glands as well as various body fluids including blood, urine, saliva and milk.<sup>27</sup>

The physiological functions of PrP<sup>C</sup> are not well understood. Previous studies have demonstrated that PrP<sup>C</sup> has diverse cellular and molecular activities including protection of cells against apoptotic and oxidative stress, cellular binding of copper ions, transmembrane signalling, the formation and maintenance of synapses, adhesion to the extracellular matrix as well as the activation, differentiation and maturation of both dendritic cells and thymocyte cells.<sup>54,94</sup>

PrP<sup>Sc</sup>, misfolded from its normal cellular counterpart (PrP<sup>C</sup>) through an unknown mechanism, differs structurally in that it is rich in beta sheets whereas PrP<sup>C</sup> contains abundant alpha helices.<sup>56</sup> Unlike common microbial pathogens, PrP<sup>Sc</sup> is devoid of nucleic acids.<sup>65,94</sup> PrP<sup>Sc</sup> is known to deposit and aggregate in the brain.<sup>56</sup> These aggregates lead to a loss of neurons. Although the molecular mechanism underlying the loss of neurons in prion diseases is not well understood, multiple pathways are likely involved. Oxidative stress, PrP trafficking alterations, neurotoxicity and apoptosis have all been suggested as contributors to neurodegeneration.<sup>1,20,31,32,52,56,89</sup>

The unusually stable conformation of PrP<sup>Sc</sup> is believed to be responsible for its resistance to conventional physical and chemical

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decontamination and inactivation procedures such as autoclaving, radiation and chemical denaturing treatments.<sup>83</sup> Such properties of PrP<sup>Sc</sup> have led to debates on the safety of current biologics and biotherapeutics, which might be contaminated with trace PrP<sup>Sc</sup>, for human use. These products may include but are not limited to: blood components for transfusion, tissue transplants, organ transplants, hormones, DNA preparations produced in vitro, vaccines, cytokines and monoclonal antibodies.<sup>24</sup> As of today, at least 196 cases of iatrogenic transmission by dura matter grafts have been reported, while at least four probable cases of PrP<sup>Sc</sup> transmission have been linked to blood transfusion.<sup>12,33</sup> Furthermore, some 200 cases of iatrogenic CJD have been associated with hormone therapy (including growth hormone and gonadotropin treatments).<sup>12,33,51</sup> As several excellent reviews have been published with respect to the iatrogenic transmission of vCJD and CJD in recent years, these topics will not be covered in depth here.<sup>12,33,39</sup> Instead, we attempt to provide a brief review about: (1) The susceptibility to PrP<sup>Sc</sup> infection of cell substrates used in the manufacture of biologics and biotherapeutics; (2) current methodologies for the detection and removal of PrP<sup>Sc</sup> in the production of biologics and biotherapeutics.

### The Susceptibility of Cell Substrates Used in the Manufacture of Biologics and Biotherapeutics to PrP<sup>Sc</sup> Infection

Numerous types of cells have been used for the production of vaccines or recombinant proteins.<sup>30</sup> The variety of cells will only continue to increase as the field of biologics and biotherapeutics expands. Several studies have been conducted to determine the susceptibilities of various cell lines to PrP<sup>Sc</sup> infection (Table 1). Some of these cells are used in the manufacture of biologics and biotherapeutics (recombinant proteins or vaccines) or treated ex vivo for gene therapy or experimental adoptive cell therapy. With cell lines or tissue samples, cells are exposed to glycerol, detergents and bovine-derived materials such as amino acids, gelatins, enzymes and blood, any of which might contain PrP<sup>Sc</sup>.<sup>23,90</sup>

In 1997 an outbreak of scrapie in sheep in Italy is believed to have been caused by a contaminated sheep brain homogenate used in a vaccine against *Mycoplasma agalactiae*.<sup>15</sup> The animals were inoculated subcutaneously with a vaccine made with ingredients derived from sheep brain and mammary homogenates.<sup>2</sup> The link between the vaccine and the cases of scrapie was made based on the patterns of mortality, incidence rates and the lesion profiles identified in the infected flocks.<sup>15</sup> Based on these factors, epidemiological studies indicated that the culprit was most likely a common source (such as a vaccine) as opposed to dietary or external animal contact.<sup>15</sup> Thus, the use of a contaminated vaccine is believed to be one of the main factors contributing to the intra- and interspecies transmission of scrapie in Italy.<sup>97</sup> It is unclear though, how the veterinary vaccine became contaminated with PrP<sup>Sc</sup>.

Since the 1970's, cells of numerous different lineages have been studied for their susceptibility to PrP<sup>Sc</sup> infections. Clarke and Haig provided the first evidence that the scrapie prion could be passaged through cell culture.<sup>16</sup> In addition, various cell lines were found to be susceptible to persistent PrP<sup>Sc</sup> infections as reported by Chesebro's and Pruisner's groups in the 1970's and 1980's.<sup>14,66</sup> One of the most studied PrP<sup>Sc</sup>-infected cells is the scrapie-infected mouse neuroblastoma cell line (ScN2a), derived from mouse neuroblastoma (N2a) cells, which was found to be persistently infected with

**Table 1 Partial list of cell lines susceptible to PrP<sup>Sc</sup> infection**

Cell line designation	Cell line	Reference
GT1-7	Hypothalamic neuronal cell line (mouse)	62, 76
L-fibroblast	Fibroblast cell line (mouse)	17
Scn2a	Neuroblastoma cell line (mouse)	14
PC12	Pheochromocytoma cell line (rat)	17, 70, 71
SMB	Mesodermal cell line (mouse)	8,16
ROV	Kidney epithelial cell line (rabbit) expressing ovine prion protein	92
MOV	Neuroglia cell line (mouse) expressing ovine prion protein	3
NIH/3T3	Fibroblast cell line (mouse)	93
L929	Fibroblast cell line (mouse)	93
SN56	Cholinergic septal neuronal cell line (mouse)	5
NSC	Fetal neural stem cells (mouse)	57
MPC	Adult multipotent progenitor cells (mouse)	57
C2C12	Myoblasts (mouse)	22
Neurosphere cell line	Central nervous system stem cell (mouse)	29
Neurons	Primary cells (mouse)	20
Astrocytes	Primary cells (mouse)	20
Dendritic cells	Primary cells (human)	72

the Rocky Mountain Laboratory (RML) mouse-adapted strain of scrapie.<sup>14</sup> Conflicting observations have been reported with respect to the biochemical or phenotypical changes in these PrP<sup>Sc</sup>-infected cells in comparison with the parent cells. For example, although some researchers reported changes in catecholamine, serotonin and noradrenaline levels in ScN2a cells, consistent reports of changes in cell phenotype or biochemistry have been lacking.<sup>53,81</sup> Furthermore, while some studies have observed morphological changes and increased proliferation in ScN2a cells, others have reported the opposite.<sup>9,53,81</sup> Nevertheless, it is noted that most of the cells investigated so far display no visible morphological or pathological changes when infected (Table 1).

So far, cell lines are only susceptible to infection by PrP<sup>Sc</sup> strains adapted from the host of the same species.<sup>67</sup> Cell lines from different mammals are often used for producing human biologics. Given that certain strains of scrapie have crossed the species barrier to infect cattle, and that BSE is believed to be responsible for vCJD infections in humans, the use of non-human cell lines in the production of biologics should be carefully monitored for prion disease.<sup>18</sup>

In general, many cell lines studied so far are not as easily infected by PrP<sup>Sc</sup> in vitro. The molecular mechanism underlying the susceptibility of cells to PrP<sup>Sc</sup> infection remains largely unknown. Several cellular receptors have been suggested to mediate the entry of PrP<sup>Sc</sup> into the targeted cells. Heparan sulphates appear to serve as a cellular receptor as demonstrated by the inhibitory effects of heparan mimetics on PrP<sup>Sc</sup> propagation in Chinese hamster ovary (CHO) cells, hypothalamus GT1-1 and neuroblastoma cells.<sup>34,36,78</sup> Yet, data obtained from studies using heterologous epithelial cells (a rabbit epithelial cell line) expressing ovine prion protein suggests heparan sulphate is not involved in PrP<sup>Sc</sup> internalization.<sup>63</sup> It seems

that the internalization of PrP<sup>Sc</sup> may have multiple routes. The non-integrin 37 kDa/67 kDa laminin receptor (LRP/LR) was identified as another cell surface receptor for PrP<sup>Sc</sup>, and is required for PrP<sup>Sc</sup> propagation in scrapie-infected cells.<sup>28,50</sup> Furthermore, Morel et al. reported that bovine PrP<sup>Sc</sup> is internalized in human Caco-2/TC7 enterocytes via LRP/LR-mediated endocytosis.<sup>60</sup> This observation supports the hypothesis that enterocytes, the major cell population of the intestinal epithelium, play a role for the uptake of prion infectious particles during oral infection. However, given the wide distribution of laminin receptor as the major glycoprotein of the basement membrane from all cell types, and the varying susceptibility of different cells types to PrP<sup>Sc</sup> infection, LRP/LR may not be solely responsible for the uptake of PrP<sup>Sc</sup>. Indeed, mounting evidence suggests that multiple cellular factors or endocytic routes could be involved.<sup>61,63</sup> For example, the presence of PrP<sup>C</sup> has been reported to be critical in supporting the propagation of PrP<sup>Sc</sup>.<sup>13,21,67,81</sup> Since only a few PrP<sup>C</sup> expressing cells can be infected by PrP<sup>Sc</sup>, PrP<sup>C</sup> itself is insufficient to confer permissiveness of cells for infectivity. It was hypothesized that PrP<sup>C</sup> could interact with exogenous PrP<sup>Sc</sup> at the cell surface in the early stage of de novo infection of epithelial cells, which might be the first place where the PrP<sup>Sc</sup> multiplication initially takes place.<sup>63</sup>

Recent studies propose that the stromal complement receptor CD21/35 could be involved in targeting PrP<sup>Sc</sup> to follicular dendritic cells (FDC).<sup>95</sup> It remains unclear as to how productive PrP<sup>Sc</sup> replicates in FDCs although they are critical for neuroinvasion of PrP<sup>Sc</sup> in the mouse model.<sup>58</sup> As CD21/35 receptors also exist in B cells, which appear to express little PrP<sup>C</sup>, the requirement for B cells to facilitate splenic PrP<sup>Sc</sup> accumulation was thought to be related to B cells supplying cytokines necessary for the maturation of FDC.<sup>41,55,95</sup> Furthermore, other receptors have been speculated to serve as cellular receptors mediating the endocytosis of PrP<sup>C</sup>, i.e., stress-inducible protein, neural adhesion molecules, lipoprotein receptor-related protein1 (LRP1).<sup>75,77,84,96</sup> How exogenous PrP<sup>Sc</sup> interacts with these receptors has not been as vigorously studied in vitro as the normal cellular PrP<sup>C</sup>. Collectively, the data documented in the literature suggests that PrP<sup>Sc</sup> may enter target cells via various routes or PrP<sup>Sc</sup> entry may be dependant on cell types.

The limited knowledge of which cellular receptors mediate PrP<sup>Sc</sup> entry hamper the understanding of what happens after PrP<sup>Sc</sup> has entered into the cell. It is unknown as to exactly what constitutes the most effective environment within cells to support PrP<sup>Sc</sup> propagation. The observation that fibroblast cells in tissue culture could be infected by PrP<sup>Sc</sup> is of particular interest as these cells express low levels of normal prion protein and are not of neuronal origin.<sup>93</sup> While results from the studies suggest that the susceptibility of a cell line to PrP<sup>Sc</sup> infection might be independent of the tissue origin or the level of normal prion proteins, the presence of PrP<sup>C</sup>, albeit at low level, appears to be necessary since knock-out of the gene encoding for PrP<sup>C</sup> by siRNA inhibits PrP<sup>Sc</sup> propagation.<sup>21</sup> In addition, mice devoid of PrP<sup>C</sup> were resistant to PrP<sup>Sc</sup> infection.<sup>13</sup> These observations suggest that PrP<sup>Sc</sup> needs to use its normal cellular counterpart (PrP<sup>C</sup>) as substrate to multiply itself. Such a notion is supported by evidences that cell susceptibility to PrP<sup>Sc</sup> infection could be increased if the cells are genetically altered, i.e. via overexpression of the normal PrP<sup>C</sup>.<sup>81</sup> The discrepancy of results in relation to the level of endogenous PrP<sup>C</sup> in support of PrP<sup>Sc</sup> propagation is conceivably due to

the differences in cell types and/or experimental systems (in vitro vs. in vivo).

Repeated attempts to establish the persistent infection of cells using PrP<sup>Sc</sup>-containing tissues that were derived directly from the diseased animals or CJD patients have not always been successful.<sup>7</sup> Exceptional cases where persistent infections could be induced include a rabbit epithelial cell line expressing ovine prion protein that has been shown to be susceptible to infection by a field scrapie isolate, a transformed deer cell line that could be persistently infected with PrP<sup>Sc</sup> derived from the brain stem of a CWD-affected mule deer and a human neuroblastoma cell line in which human prions could be propagated.<sup>47,68,92</sup> Nevertheless, the exact cellular and molecular mechanisms underlying the incapability of some PrP<sup>Sc</sup> strains to infect cells of a different species, a phenomenon known as the 'species barrier', and the improved infectivity of PrP<sup>Sc</sup> for cultured cells following multiple passages in rodents remain to be elucidated.<sup>19,59</sup>

### Current Methodologies for the Detection and Removal of PrP<sup>Sc</sup> in the Manufacture of Biologics and Biotherapeutics

A variety of methodologies intended to identify prion contaminants in biologics and biotherapeutics have been developed in recent years and these tests vary in sensitivity and specificity. Detection of PrP<sup>Sc</sup> can be accomplished either by in vivo bioassays or in vitro biochemical/immunological assays. Bioassays usually involve injecting samples intracranially into rodents followed by monitoring for the development of neurological disease; however, the method is often very time consuming and costly. New biochemical and immunological assays including the cell blot, Western blot (WB) and ELISA.<sup>10,37,64,73,80</sup> Cell blotting has been shown to be a sensitive method for the detection of PrP<sup>Sc</sup> in infected cells and involves transferring cells directly to a nitrocellulose or polyvinylidene difluoride membrane before subjecting the membrane to standard WB protocols.<sup>10</sup> Quantification of infected cells by cell blot could also be achieved through the use of automated counting equipment.<sup>42</sup> It is encouraging to note that this is as sensitive as the mouse bioassay but much faster and less expensive.<sup>42</sup> This system is reported to be limited to mouse-adapted scrapie strains but could still be adapted for other prion proteins. Conformation-dependent immunoassay was also judged to be as sensitive as the cell blot.<sup>6</sup> Western Blot (W.B.), albeit less sensitive, has been used extensively to detect PrP<sup>Sc</sup> and to confirm results obtained from other tests because the "signature" isoforms of PrP<sup>Sc</sup> (monoglycosylated, diglycosylated and unglycosylated) can be clearly identified.<sup>10</sup>

The sensitivity of some of these immunoassays has been substantially improved with an extra step of protein concentration prior to SDS-PAGE separation and W.B. transfer. Available antibodies generally recognize both PrP<sup>Sc</sup> and PrP<sup>C</sup> so samples are often treated first with proteinase K (PK) to remove PrP<sup>C</sup>, followed by concentration of the remaining PrP<sup>Sc</sup> to improve sensitivity. Protein concentration is particularly necessary for tissue and/or cell samples containing low concentrations of PrP<sup>Sc</sup>.<sup>10,93</sup> There are several procedures being used to enrich PrP<sup>Sc</sup> from tissue and cell samples; including, precipitation by phosphotungstic acid (PTA), ethanol, methanol or trichloroacetic acid (TCA), and concentration by ultracentrifugation.<sup>37,93</sup> TCA precipitation, in combination with urea denaturation, has been particularly effective in obtaining higher yields of abnormal proteins.<sup>48</sup> Recently, a method with the capability of cyclically

amplifying the misfolded prion protein (protein misfolding cyclic amplification, PMCA) was shown to be very sensitive in detecting prion proteins in hamster blood samples (containing PrP<sup>Sc</sup>) and human brain samples (containing vCJD).<sup>40,73</sup> If optimized, validated and automated, this method could have great potential for high throughput screening of blood products or other types of biological products for human use since it is conceptually analogous to polymerase chain reaction cycling.

An array of methodologies exists that are intended to safeguard biologics and biotherapeutics against prion contamination. Techniques to remove or reduce prions in blood or plasma products involve a array of protocols.<sup>23</sup> For example, techniques such as the depletion of leukocytes in the preparation of packed red blood cells and a variety of protein purification and fractionation procedures in the manufacture of plasma products have been implemented. Some of these methods traditionally used in the manufacturing of plasma products have been found to substantially reduce prion-associated infectivity. For instance, ethanol fractionation to purify albumin and immunoglobulin has been shown to separate prion proteins into precipitates, which are subsequently discarded during the manufacturing process.<sup>49</sup> This partitioning step, in conjunction with removal of the precipitates by depth filtration, resulted in significant clearance of PrP<sup>Sc</sup> in plasma intermediates spiked with TSE-infected material, with a reduction in infectivity ranging from 1–6 logs.<sup>49,87</sup> In addition to ethanol precipitation, ion-exchange chromatography has been reported to reduce TSEs by 2–4 logs, while combined chromatography procedures (DEAE Sepharose, CM Sepharose and Macro-Prep High Q chromatographic columns) were reported to reduce the amounts of infectious prions to levels undetectable by bioassays.<sup>25,26,86</sup> Another technique, nanofiltration, showed a decrease of infectious TSE materials by approximately three logs.<sup>23,46,82</sup> While the effectiveness of these methods in removing prion proteins spiked in the testing samples is convincing, questions remain unanswered as to the equivalence of the spiked samples to the real prions present in samples of biologics and biotherapeutics and the detection limits of the current methods. In addition, the minimal infectious dose of PrP<sup>Sc</sup> to cause the disease in humans is yet to be determined.

## Concluding Remarks

Tremendous progress has been made in our understanding of TSE agents and the related diseases following the identification of prion proteins. The fact that prion proteins can potentially infect and be sustained in a variety of cells and tissues, complicated by a lack of morphological change following PrP<sup>Sc</sup> infection, require that robust measures be put in place to ensure the safety of biologics and biotherapeutics. While evidence appears to be strong that scrapie transmission has taken place through the use of veterinary vaccines, there has been no report suggesting that vCJD transmission is linked to the use of human vaccines.<sup>14</sup> Yet, it is of note that the incubation periods of human prion diseases could be as long as decades.<sup>18</sup> Therefore, one may safely conclude that the question of TSE transmission by biologics and biotherapeutics remains open. It is encouraging though, that various methods have been proven to be effective in removing PrP<sup>Sc</sup> from the manufacturing process of biologicals in experimental studies. However, many questions remain unanswered. These include the resemblance of the “spiked” samples

to the real PrP<sup>Sc</sup>-containing intermediate products in the manufacturing process, the minimal dose of PrP<sup>Sc</sup> required to cause TSE in humans, and the ability of current methods to identify such minimal inoculums of PrP<sup>Sc</sup>. While a variety of cellular receptors have been implicated to mediate the endocytosis of PrP<sup>Sc</sup> or PrP<sup>C</sup>, additional cellular factors remain elusive. The identified receptors often allow entry of a plethora of substances or exist in all cells, which cannot explain the differences of susceptibility of various cell types to PrP<sup>Sc</sup> infections. Of note is the fact that fibroblast cells are susceptible to PrP<sup>Sc</sup> infection since these cells have been used as feeder cell layers for keratinocyte cultures in cutaneous gene-therapy applications.<sup>69</sup> Furthermore, it is still unclear as to whether ex vivo treatments of cells with various cytokines or stimuli in exploratory cell or gene therapies might make these cells more susceptible to prion infection, given that cytokines could affect PrP<sup>Sc</sup> accumulation and that the susceptibility of the cells to prion infection might be independent of tissue origin and the level of endogenous prion proteins.<sup>18,41,55,95</sup> To answer these questions, more in-depth studies will be needed, especially at a time when the number and the diversity of cell substrates used for the manufacture of biologics and biotherapeutics or as gene/cell therapies will only increase in the years to come.

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