Deer Prion Proteins Modulate the Emergence and Adaptation of Chronic Wasting Disease Strains.

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Abstract

Transmission of chronic wasting disease (CWD) between cervids is influenced by the primary structure of the host cellular prion protein (PrP\textsuperscript{C}). In white-tailed deer, \textit{PRNP} alleles encode polymorphisms Q95G96 (\textit{wt}), Q95S96 (\textit{S96}) and H95G96 (\textit{H95}) that differentially impact CWD progression. We hypothesize that transmission of CWD prions between deer expressing different allotypes of PrP\textsuperscript{C} modifies the contagious agent affecting disease spread. To evaluate the transmission properties of CWD prions derived experimentally from deer of four \textit{PRNP} genotypes (\textit{wt/wt}, \textit{S96/wt}, \textit{H95/wt} or \textit{H95/S96}), transgenic (tg) mice expressing \textit{wt} (tg33) or \textit{S96} (tg60) alleles were challenged with these prion agents. Passage of deer CWD into tg33 mice resulted in 100% attack rates, with H95/S96 CWD having significantly longer incubation periods. Disease signs, neuropathological and PrP-res profiles in infected tg33 mice were similar between groups, indicating a prion strain (Wisc-1) common to all CWD inocula was amplified. In contrast, tg60 mice developed prion disease only when inoculated with the H95/wt and H95/S96 CWD allotypes. Serial passage in tg60 mice resulted in adaptation of a novel CWD strain (H95\textsuperscript{+}) with distinct biological properties. Transmission of first-passage tg60CWD-H95\textsuperscript{+} isolates into tg33 mice, however, elicited two prion disease presentations consistent with a mixture of strains associated with different PrP-res glycotypes. Our data indicates that \textit{H95}-\textit{PRNP} heterozygous deer accumulated two CWD strains, whose emergence was dictated by PrP\textsuperscript{C} primary structure of the recipient host. These findings suggest CWD transmission between cervids expressing distinct PrP\textsuperscript{C} molecules results in generation of novel CWD strains.
Importance

CWD prions are contagious among wild and captive cervids in North America and in Korea. We present data linking the amino acid variant Q95H in white-tailed deer cellular prion protein (PrPC) to the emergence of a novel CWD strain (H95+). We show that, upon infection, deer expressing H95-PrPC molecules accumulated a mixture of CWD strains selectively propagated depending on the PRNP genotype of the host in which passaged. Our study also demonstrates that mice expressing the deer S96-PRNP allele, previously shown to be resistant to various cervid prions, are susceptible to H95+ CWD prions. The potential for generation of novel strains raises the possibility of an expanded host range for CWD.
Introduction

Chronic wasting disease is an emerging prion disease or transmissible spongiform encephalopathy (TSE) of cervids, affecting free-ranging white-tailed deer (Odocoileus virginianus), mule deer (Odocoileus hemionus), elk (Cervus elaphus canadensis) and moose (Alces americanus sp.) (1, 2). CWD occurs in captive herds of these species in North America and in red deer (Cervus elaphus sp.) and sika deer (Cervus Nippon sp.) in Korea (1, 3, 4). Reindeer (Rangifer tarandus sp.), also known as caribou, are susceptible to experimental infection (5).

TSEs are slowly progressive, fatal neurodegenerative disorders with no effective treatment or vaccine available. Neuropathological changes include prion protein deposits, spongiform degeneration, neuronal loss and astrogliosis. These hallmarks are diagnostic for CWD in cervids, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) as well as kuru, iatrogenic (iCJD) and variant Creutzfeld-Jakob disease (vCJD) in humans (6-10).

Pathogenesis of TSEs is associated with abnormal PrP^Sc (or PrP^{CWD} for cervid infections), whose ability to propagate, persist and trigger neuropathology requires the expression of host-PRNP-encoded cellular prion protein (PrP^C). Prion propagation involves the post-translational misfolding of normal cellular PrP molecules into pathognomonic, generally protease-resistant (PrP-res), transmissible protein conformers that progressively accumulate in brain and other tissues (11-14). The primary structure of PrP^C influences host susceptibility to infection, its
The difficulty of prion transmission from one species to another is defined as the species barrier, or, between individuals of the same species with different PRNP genotypes, the transmission barrier, and is influenced by the primary structure of the recipient’s PrPC (15, 17, 19, 20, 25, 26). This barrier does not necessarily render the host refractory to infection and is impacted by the invading prion strain (20, 21, 26, 27). Prions can exhibit strain diversity. Strains are distinguished based on their host range, clinical presentation, disease progression, neuropathological and PrP biochemical profiles (28-31). Propagation of prion strains is dependent on both the PRNP genotype of the recipient as well as the properties of the invading agent (27, 32). For example, sheep expressing the V136-R154-Q171-PrPC (GenBank accession # AJ567988) are most susceptible to classical scrapie, while sheep with distinct PRNP genotypes have reduced susceptibility (33-35). The strain of the agent also plays a role, as sheep expressing A136-R154-R171 (AJ567985) or A136-H154-Q171-PrPC (AJ567983), although relatively resistant to classical scrapie, are susceptible to atypical scrapie (31, 36). Similarly, the PrPC primary structure and the invading agent modulate human susceptibility to prion infection; polymorphisms at codon 129 affect susceptibility to vCJD, Kuru and iCJD (20, 21, 26, 27, 37-40), while G127V renders carriers resistant against Kuru (41).

In CWD-enzootic regions of North America, transmission occurs between cervids expressing heterologous PrPC molecules (PrP^C-allotypes; 18). Analysis of PRNP allelic frequencies in wild
and captive white-tailed deer identified two PrP\textsuperscript{C} polymorphisms Q95H and G96S that impact susceptibility to CWD (42-44) (GenBank accession # AF156185, AF156184 and AY275711). Homozygous wt (Q95G96) deer are most susceptible to CWD and have relatively short incubation periods. In contrast, deer heterozygous for S96/wt, H95/wt and H95/S96 alleles had extended incubation periods suggesting S96-PrP\textsuperscript{C} and H95-PrP\textsuperscript{C} impact CWD prion propagation (45). Miller et al. (46) reported similar observations in experimentally challenged S96/wt and S96/S96 deer compared to wt/wt white-tailed deer and mule deer. To further explore the diversity of CWD strains and the consequences of propagation in deer expressing different PrP\textsuperscript{C} primary structures, CWD brain homogenates from white-tailed deer of different PRNP genotypes (wt/wt, S96/wt, H95/wt or H95/S96; 45) were inoculated into transgenic mice expressing deer wt or S96 alleles (47, 48). Our data shows that CWD prions passaged in deer expressing H95-PrP\textsuperscript{C} have altered their transmission properties. H95/wt and H95/S96 CWD allotypes efficiently triggered prion disease in tg mice with S96-PRNP genotypes, leading to the identification and adaptation of a novel CWD strain. Transmission of first passage tg60CWD-H95\textsuperscript{+} prions into tg33 mice resulted in two distinct prion disease phenotypes, which resemble those observed after primary passage of H95-PrP heterozygous deer CWD in both tg lines.

Materials and Methods

Deer CWD inocula

Four CWD agents, brain homogenates (BH) of 10% or 1% weight per volume (w/v) in phosphate buffered saline were used for transmission studies (45). The inocula are designated based on their specific PRNP genotypes. CWD brain homogenates were obtained from orally infected
white-tailed deer expressing different PrP C molecules: homozygous Q95G96 (wt/wt), heterozygous Q95S96/wt (S96/wt), heterozygous H95G96/wt (H95/wt) and H95G96/Q95S96 (H95/S96) (45). Brain homogenate from an uninfected white-tailed deer was used as a negative control. Frozen sagittal brain halves were homogenized (blended) to 20% (w/v) in cold phosphate buffer (1.3 M NaCl, 70 mM Na2HPO4·2H2O, 30 mM NaH2PO4·2H2O, pH 7.4), aliquoted and stored at -80 °C. Subsequently, aliquots were homogenized in a 50 mL syringe by passage through needles of different size (18G to 21G).

Transmission studies in transgenic mice

Animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Health Sciences Animal Care and Use Committee of the University of Alberta ACUC. Bioassays were performed with transgenic mouse lines expressing deer wt (tg33+/+ and tg33 +/-) or S96 PRNP alleles (tg60 expression fold ratio 0.7 < tg33 +/-) (47, 48). Weanling pups were inoculated intra-cerebrally with 30 µL of deer CWD brain homogenates. Animals were monitored for appearance of clinical signs and disease progression. Individual incubation periods are expressed as days post-inoculation (dpi) and were calculated from the time mice were inoculated until clinical disease was established. The distribution of tg33 mice incubation periods was compared between groups using Kruskal-Wallis test with Dunn’s multiple comparison post-test (P < 0.05). Survival times post inoculation between tg60 mice inoculated with H95/wt and H95/S96 deer CWD allotypes were compared using Mann-Whitney test (P < 0.05). The statistical analysis of transmission experiments was performed with GraphPad Prism® Version 5.04.
TgCWD isolates derived from tg60 mice infected with H95/wt or H95/S96 deer CWD allotypes were used for syngeneic and allogeneic passages. One tg60CWD-H95/wt isolate (10% w/v) was transmitted in tg33 and tg60 mice. A tg60CWD-H95/S96 isolate (10%, 1%, 0.0001% w/v) was also passaged into both tg lines.

**Histopathological analysis**

Brain tissues from at least 5 (5 to 11) tg-mice per inoculum-group were formalin-fixed and paraffin-embedded for histopathological analysis. From each inoculum-group of animals, sagittal brains sections were obtained from 2 to 4 mice and coronal brain sections were obtained from 3 to 7 mice. Six consecutive brain slides of both sagittal and coronal sections (1 brain = 4 coronal sections) were examined as follows: 2 slides (4-6 µm thick) were stained with haematoxylin and eosin (H&E) to evaluate for spongiform degeneration and the other 4 slides were immunostained for PrP\textsuperscript{CWD} deposition and GFAP-positive astroglia. The sagittal paramedian brain sections were 0.36-0.60 mm lateral from the brain midline. All immunostaining experiments included positive (CWD-positive tissue) and negative (mock-infected controls). Differences in PrP\textsuperscript{CWD} deposition patterns could exist in areas of the CNS that were not examined. For the purpose of comparison, structure identification was performed in H&E stained slides following the mouse brain atlas (49).

Lesion profile analysis was performed using brain coronal sections as described (50). The lesion profile scores for first passage in tg33 mice were obtained from 3 to 7 mice per inoculum-group.
For the CWD-affected tg60 mice, lesion profiles were obtained by scoring 4 mice per inoculum-group. The density of spongiform lesions in nine grey matter areas from brains of prion disease-affected mice were blind-scored by three independent observers. The scores are reported as the mean ± standard deviation.

PrP\textsuperscript{CWD} deposits were visualized by immunostaining using anti-PrP monoclonal antibodies BAR224 or 8G8 (bertinpharma, Spi-Bio\textsuperscript{®}) (0.2 µg/ml diluted 1:2000 or 1:100 respectively). Briefly, brain slides were pre-treated with high-pressure autoclaving (2.1 bar) for 30 minutes in citric acid (10 mM) pH 6.0 at 121ºC degrees, followed by treatment with 98% formic acid for 30 minutes and 4 M guanidine thiocyanate for 2 hours at room temperature. Astrogliosis was evaluated by immunostaining of glial fibrillary acidic protein using an anti-GFAP antibody (BD Biosciences) (0.5 mg/ml diluted 1:1000), after hydrated autoclaving for epitope exposure. Immunohistochemical detection was achieved with biotinylated secondary antibodies according to the manufacturer’s instructions (DAKO ARK\textsuperscript{™}). Tissue sections were scanned with a Nanozoomer 2.0RS (©Hamamatsu Photonics) and analyzed using Nanozoomer digital pathology software (©Hamamatsu Photonics).

**Immunoblot analysis**

Brain tissues from tg mice were homogenized to 10% (w/v) in sterile water using disposable syringes and needles of decreasing diameters (18G to 21G), aliquoted and stored at -80ºC. Brain homogenate protein content was determined using MicroBCA assay kit (Life technologies\textsuperscript{™}). For the proteinase digestion reactions, 50-70 µg total protein (1-1.4 mg/mL final protein sample
concentration) was treated with 150 µg/mL of proteinase K (Life Technologies™) for 45 minutes at 37°C. Reactions were terminated by boiling samples in 2.5X Laemmli buffer (150 mM Tris-Cl pH 6.8, 0.5% bromophenol blue, 25% glycerol, SDS 5% w/v and 12.5% β-mercaptoethanol) at 95°C for 10 minutes. Samples (10-15 µg) were resolved on 12% NuPAGE Bis-tris gels (Life technologies™) and transferred onto PVDF immobilon-P membranes (Millipore). Membranes were blocked for 1 hour at room temperature with 5% (w/v) non-fat dry milk in Tris-buffer saline containing 0.1% (v/v) Tween-20 (TBST). Detection was performed using primary monoclonal antibody BAR224 (bertinpharma, Spi-Bio®; 0.2 µg/ml diluted 1:10000 in 5% (w/v) non-fat dry milk-TBST solution) or 8G8 (bertinpharma, Spi-Bio®; 0.2 µg/ml diluted 1:2000), secondary HRP-conjugated goat anti-mouse IgG antibody and chemiluminescent substrate (Life technologies™; diluted 1:10000). Images were acquired on X-ray film (Super Rx Fujifilm™). PrP-res glycoform ratios were determined using three animals per inocula group; samples were resolved by western blot and detected with X-ray film. Quantification of PrP-res ratios was performed using Image J software (NIH Gov). Independent triplicate measurements of each sample-group were averaged and compared using GraphPad Prism® Version 5.04.

**Results**

**Transmission of experimental CWD into tg-deer-PRNP mice**

To evaluate the transmission properties of CWD prions derived from experimentally infected white-tailed deer of different PRNP genotypes (45), tg33 (expressing deer wt-PrP(C)) ortg60 (expressing deer S96-PrP(C)) mice (47, 48) were inoculated intracerebrally with deer CWD brain...
homogenates of 10% or 1% (w/v). All CWD inocula (wt/wt, S96/wt, H95/wt, H95/S96) resulted in clinical prion disease in mice expressing deer wt-PrP\(^C\) (tg33\(^{+/+}\) and tg33\(^{+/-}\)) (Fig. 1 and Table 1). Mice presented with similar disease signs including hyperactivity, kyphosis, ataxia and myoclonus. Clinical signs variably progressed into a general weakening at which time the animals were euthanized. Tg33 mice inoculated with H95/S96 CWD had significantly longer incubation periods than mice receiving the other CWD inocula (Kruskal-Wallis, Dunn’s post-test \(P < 0.05\)) (Fig. 1A, 1C and 1D). No significant differences in incubation period were observed between tg33 mice inoculated with S96/wt, H95/wt or wt/wt CWD inocula (Kruskal-Wallis, Dunn’s post-test \(P > 0.05\)) (Fig. 1).

In contrast to the susceptibility of the tg33 line, mice expressing deer S96-PrP\(^C\) (tg60) developed clinical disease only when inoculated with CWD prions derived from deer expressing the \(H95-PRNP\) allele (Fig. 1B). Mice inoculated with H95/S96 and H95/wt CWD allotypes were clinically positive for prion disease between 359-473 dpi. Affected mice became lethargic with myoclonus, kyphosis, labored breathing and atactic gait characterized by limb weakness. Incubation periods were significantly different between tg60 mice inoculated with H95/S96 or H95/wt CWD agents (Mann-Whitney, \(P < 0.05\)). S96-PrP\(^C\) mice inoculated with wt/wt and S96/wt CWD did not develop prion disease at > 700 days post-inoculation.

**Neuropathology of tg-deer-PRNP mice infected with CWD**

To define the neuropathological hallmarks and assess differences between groups of mice inoculated with different CWD inocula, sagittal and coronal brain sections were examined.
histologically for spongiform change and immunohistochemically for PrP\textsuperscript{CWD} aggregates and GFAP-positive astroglia (Fig. 2).

CWD-infected tg33 (wt-PrP\textsuperscript{C}) mice presented with extensive pathology in various brain regions and were characterized by neuronal loss, spongiform change, widespread accumulation of PrP\textsuperscript{CWD} aggregates and astrogliosis (Fig. 2A-H). The distribution of pathological changes in the brain (i.e PrP\textsuperscript{CWD} distribution) was similar between mice inoculated with the four CWD inocula (Fig. 3A-G). The average spongiform change score of various brain structures was similar between infected tg33 mice (Fig. 2Q). In general, the lesions observed (vacuolation and PrP\textsuperscript{CWD} accumulation) in the forebrain and cerebellum agree with previous results obtained with this transgenic mouse line after infection with CWD from other sources (47). Additionally, the granular layer of the cerebellum had areas of neuronal loss, where dense PrP\textsuperscript{CWD} aggregates surrounded by GFAP positive astrocytes were revealed in consecutive tissue sections (Fig. 2F-G). Spongiform change and cell death in the cerebellum of tg33 mice was less conspicuous in the molecular layer and more abundant in the white matter and the Purkinje cell layers (Fig. 2H). Infection of tg33 mice resulted in more prominent PrP\textsuperscript{CWD} accumulation in the corpus callosum (Fig. 2A-B) than described in other studies (47).

Susceptibility of S96-PrP\textsuperscript{C} (tg60) mice to CWD infection was strongly influenced by the invading CWD allotype. All tg60 mice exposed to H95/wt or H95/S96 CWD developed clinical prion disease with similar neuropathology (Fig. 2I-P, R and Fig. 3J-K). The distribution and severity of neuropathological changes observed in diseased tg60 mice infected with H95\textsuperscript{+} deer
CWD allotypes followed a consistent lesion pattern (Fig. 2I-P, R and Fig. 3J-K). Spongiform degeneration and abnormal S96-PrP\textsuperscript{CWD} aggregates were localized in the caudoputamen, the corpus callosum, and extended down the septum to the diagonal band nucleus (Fig. 3K). In the cerebral cortex and hippocampus, both vacuolation and PrP\textsuperscript{CWD} deposition were of milder intensity than in other brain areas, however, immunohistochemical staining revealed the presence of small, punctate S96-PrP\textsuperscript{CWD} aggregates at higher magnification (Fig. 2K). Pathological changes were more severe in various regions of the thalamus, including the medial-dorsal, ventral-medial and ventral anterior-lateral thalamic nuclei (Fig. 2I-J, L-M and Fig. 3J), also involving the zona incerta, cerebral peduncle, sub-thalamic and hypothalamic nuclei (Fig. 2I-J and Fig. 3J). In the midbrain, lesions were localized in the substantia nigra adjacent to the ventral tegmental area, extending to periaqueductal gray and adjacent structures, including the raphe nucleus, mesencephalic reticular formation and superior cerebellar peduncle (Fig. 2I and Fig. 3K). Pathology was also observed in the hindbrain, affecting various regions including the median raphe nucleus and pontine reticular nucleus (Fig. 3K). In cerebellum, spongiform change was most prominent in the white matter; however, small vacuoles were also observed in the molecular, Purkinje and granular layers, the latter showing minor loss of granular neurons (Fig. 2N). PrP\textsuperscript{CWD} staining revealed either diffuse deposits (lightly stained) or larger confluent aggregates in the cerebellar nuclei and the granular layer (Fig. 2I, P and Fig. 3K).

A few (3/28 and 3/31) non-clinical tg60 (S96-PrP\textsuperscript{C}) mice infected with wt/wt and S96/wt CWD had detectable prion aggregates at >700 days post inoculation (Fig. 3H-I), highlighting the low efficacy of these CWD agents to establish infection in this transgenic line. Accumulation of
PrP\textsuperscript{CWD} aggregates in these particular mice did not follow PrP\textsuperscript{CWD} distribution patterns described in the other mice.

**PrP-res glycotypes in transgenic mice expressing deer-PrP\textsuperscript{C}**

Distinct PrP-res isoforms have been associated with different prion strains (28, 51, 52). PrP-res can vary in molecular weight, glycoform ratio and other biochemical properties related to the structural stability of the abnormal PrP conformers (53). These properties have been interpreted as conformational differences in the structures of the misfolded PrP molecules that encode the information that defines different prions strains (28, 51-53). To compare PrP-res in mice infected with different CWD inocula, brain homogenates were digested with proteinase K and analyzed by western blot using anti-PrP monoclonal antibodies 8G8 (Deer PrP 98-113) or Bar224 (Deer PrP 144-154). All clinically affected tg33 mice were PrP-res positive; no differences were observed with respect to molecular weight and glycoform pattern (Fig. 4). Although the electrophoretic profile of PrP-res from clinically affected tg60 mice was similar between mice inoculated with H95/wt or H95/S96 CWD allotypes, this PrP-res type was distinct from that observed in tg33 mice. Gel migration of PK cleavage products indicates a lower molecular weight of S96-PrP-res than observed with wt-PrP-res (Figs. 4 and 5). PrP-res was not detected in brain homogenates from tg60 mice inoculated with wt/wt and S96/wt CWD allotypes at 700 dpi.

**Serial transmission of passage 1 tg60CWD-H95\textsuperscript{+} isolates into tg-deer-PRNP mice**

To evaluate the transmission properties of tg60 (S96-PrP\textsuperscript{C})-passaged CWD prions, we inoculated these isolates into both the tg33 and tg60 transgenic lines. Serial transmission of first passage
tg60CWD-H95+ isolates back into tg60 mice (syngeneic passage) resulted in reduction of the incubation periods (Fig. 5A and Table 1). Disease signs, biochemical PrP-res glyctype and neuropathology resemble the first passage characteristics (Fig. 5B-D).

Passage of brain homogenates from tg60CWD-H95+ isolates into tg33 mice (allogeneic passage) resulted in two different prion disease presentations. After exposure to 10% (w/v) brain homogenate from the tg60CWD-H95/wt isolate, mice had extended incubation periods compared to the first passage of this deer CWD allotype in tg33 mice (Fig. 6A and Table 1). Disease signs and pathological hallmarks were similar to those described during the first passage of deer CWD in tg33, characterized by hyperactivity, widespread distribution of aggregates in the brain and high molecular weight PrP-res glyctype (Fig. 6B-C). Transmission of tg60CWD-H95/S96 prions into tg33 mice resulted in divergent prion disease phenotypes. Inoculation of 10% brain homogenates resulted in extended incubation periods with some mice developing disease signs and pathology characteristic of tg33 infected with deer CWD while others resembled the disease phenotype described for tg60 mice (Fig. 6). Evaluation of proteinase K resistant-PrP in brain homogenates from affected mice revealed PrP-res glyctotypes of distinct molecular weights (Fig. 6B). Passage of 1% and 0.0001% (w/v) brain homogenates resulted in further extension of the incubation period and increased the abundance of mice with lethargic presentation (tg60-like), accompanied by accumulation of low molecular weight PrP-res glyctype and localized deposition of PrP aggregates (Fig. 6).

Discussion
To explore the transmission properties of CWD prions derived from white-tailed deer of four different PRNP genotypes (45), we inoculated transgenic mice expressing deer prion proteins associated with susceptibility (tg33 = wt-PrPC) or resistance (tg60 = S96-PrPC) to CWD infection (47, 48). Transmission of deer H95/wt and H95/S96 CWD allotypes resulted in the emergence of a distinct CWD strain (H95+). This novel prion agent was identified when CWD brain homogenates from deer containing H95-PrP molecules were transmitted into tg60 mice. Passage of these deer homogenates into tg33, however, resulted in a prion disease phenotype indistinguishable to that observed following infection with wt/wt and S96/wt CWD. The ability of H95+ deer CWD to cause clinical prion disease in tg60 mice, which have been shown to be resistant to other CWD isolates, indicates that a new strain has emerged (45, 47, 48). Our data shows that passage of CWD (wt/wt pool) through H95/wt and H95/S96 deer resulted in a mixture of at least two CWD strains, distinguishable based on the tg-deer-PRNP genotype in which they were propagated.

All deer CWD samples, upon first passage (P1) into tg33 mice, resulted in similar disease signs, PrP-res and neuropathological features, suggesting expression of wt-PrPC favored the propagation of a CWD strain (prion conformer) common to all inocula. We refer to this agent as “Wisc-1”. Our results suggest “Wisc-1” is similar to CWD-1 described by Angers et al. (54). The white-tailed deer sample analyzed in the Angers et al. study was a wt/wt CWD positive isolate from Wisconsin. Whether Wisc-1 and CWD-1 are identical is difficult to ascertain as the white-tailed deer agents were passaged in different transgenic mice. The H95+ CWD strain differs from Wisc-1, CWD-1 and CWD-2 strains (54, 55).
We found that inoculation of H95/S96 CWD into tg33 mice resulted in significantly different incubation periods compared to other CWD allotypes. The absence of wt-PrP\textsuperscript{CWD} in this inoculum and, thus, the lack of homologous prion conversion likely contributed to the prolonged incubation period. The presence of more than one prion conformer within this inoculum may result in competition between agents, leading to propagation interference and extension of the incubation periods (56-59).

Incubation periods were not statistically different between tg33 mice infected with wt/wt, H95/wt or S96/wt CWD. Additionally, all tg33 mice presented the same prion disease phenotype irrespective of the CWD inocula they received. One possible interpretation for the phenotypic similarities observed between tg33 mice is the “Wisc-1” conformers have adaptive advantage in hosts (either in deer or tg mice) expressing wt-PrP\textsuperscript{C}. Differences in incubation periods between H95/wt and H95/S96 infected tg33 mice suggest that the PrP\textsuperscript{C} sequence in these deer impacted the proportion of accumulated CWD strains. It has previously been demonstrated in hamster co-infection experiments that the ratio of strains in a prion mixture influences the emergence of the fastest replicating or dominant strain (56, 57, 59).

The differential susceptibility to prion infection is modulated by PrP\textsuperscript{C} amino acid variability and the invading prion strain (15, 17, 20, 21, 25, 26, 60). Both natural and experimental infections support the association of the S96-PrP\textsuperscript{C} with reduced susceptibility and slower progression of CWD (3, 42-48, 55). Tg60 (S96-PrP\textsuperscript{C}) mice were previously shown to be resistant to CWD.
isolates from different cervid species (47, 48). In our study, tg60 mice inoculated with wt/wt and
S96/wt did not present with clinical disease after > 700 dpi, however, mice receiving H95/wt and
H95/S96 CWD allotypes developed disease signs and presented consistent neuropathology and
PrP-res glycotypes. Second passage of tg60CWD-H95+ isolates into tg60 mice resulted in
reduction of the incubation periods and similar phenotypic characteristics.

Allogenic transmission of the first passage tg60CWD-H95+ isolates into tg33 resulted in mice
developing two distinct prion disease phenotypes resembling the “Wisc-1” and “H95+” prion
strains. While some animals presented with hyperactivity and displayed widespread
accumulation of disease associated-PrP in brain as well as high molecular weight PrP-res, others
were lethargic with localized PrP\textsuperscript{CWD} deposits and a distinct PrP-res glycotype. Transmission of
diluted tg60CWD-H95+ inoculum resulted in more mice presenting the tg60-like phenotype. This
suggests the tg60 donor mouse, which preferentially amplified the H95+ strain, contained a
persistent Wisc-1 fraction that was amplified upon passage at high dose (10% Bh) in tg33 mice.
Transmission of lower doses of the inoculum, likely altered the proportion of the two-prion
conformers, favoring the propagation of the H95+ strain. A similar outcome is observed when
dilutions of TME were passaged in hamsters, resulting in the isolation of the Hyper and Drowsy
strains (56). Transmission of tg60CWD-H95+ isolates into tg33 mice indicates that individual
tg60 mice accumulated CWD agent mixtures. Although prion transmission experiments in tg
mice do not always recapitulate what is observed in the wild (i.e., tg60 mice are resistant to a
number of different CWD strains whereas 96S homozygous deer are naturally infected), natural
scrapie and CWD isolates have been shown to contain strain mixtures that can be differentiated by serial passage in mouse models or by histopathological and biochemical analyses (54, 61-63).

Deer with S96-PRNP alleles can be infected with CWD but have extended preclinical periods suggesting they could shed infectivity over longer periods of time compared to wt homozygous deer (45, 46, 48). Additionally, in CWD endemic areas, white-tailed deer with S96-PRNP alleles likely have a fitness advantage compared to the more susceptible deer genotypes and, as a result, the “resistant” allele may become more abundant in the population (64). An increase in the S96-PRNP allele frequency could also affect the potential for selection of CWD strains able to infect resistant genotypes. Likewise, other PRNP alleles associated with extension of the CWD pre-clinical phase, such as H95-PRNP, could also be subjected to disease-driven increase in white-tailed deer populations. Our transmission data shows that deer expressing H95-PrP accumulate a CWD strain capable of infecting S96-PRNP genotypes unlike other CWD agents. An increase in the frequency of H95-PRNP would also increase the likelihood of emergence of H95+ CWD prions. Our data suggest that white-tailed deer expressing different PrP C allotypes can accumulate and transmit CWD strain mixtures.

CWD epizootics involve multiple factors including the contagious nature of the agent, host-pathogen interactions, agent “strains” and cervid population genetics. Our data indicates CWD strain emergence is modulated by amino acid polymorphisms in the cervid PrP. CWD transmission between hosts with different PRNP genotypes (65) has the potential to generate and select novel prion conformations. Deer expressing H95-PrP C accumulate CWD prions with
different transmission properties as exemplified by its ability to infect "resistant" $S96-PRNP$ mice. Finally, our study highlights the importance of characterizing the diversity of CWD strains and their potential for interspecies transmission, as various mammalian species are susceptible to experimental CWD infection (66-69). Although several lines of evidence suggest that humans are resistant to CWD prions (70-73), not all CWD strains have been tested for their zoonotic potential. Our results demonstrating that H95+ deer CWD have different transmission properties than CWD prions composed of wt- or S96-PrP suggest the need for evaluation of the transmissibility of CWD allotypes.

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References


Eight prion strains have PrP(Sc) molecules with different conformations. Nat Med 4:1157-1165.


**Figure Legends**

**Fig. 1. Transmission of CWD allotypes into transgenic mice expressing deer wt or S96 PrP<sup>C</sup>.** (A) Susceptibility of tg33 (wt-PrP<sup>C</sup>) mice to infection with 10% (w/v) CWD brain homogenates (Bh). (B) S96-PrP<sup>C</sup> (tg60) mice developed clinical prion disease only when inoculated with CWD prions derived from deer expressing the H95-PrP<sup>C</sup>. Mice inoculated with wt/wt (open circles) or S96/wt (open squares) CWD did not show with clinical signs. Crossed symbols represent animals euthanized due to intercurrent disease. (C and D) Comparison of
incubation periods in tg33+/+ and tg33+/- mice. Bars with asterisks indicate significant group
differences (Kruskal-Wallis test, Dunn’s multiple comparisons post-test P < 0.05). Mann-
Whitney test (P < 0.05) was used to compare the distribution of incubation periods in tg60 mice.

Fig. 2. Neuropathology of tg-deer-PRNP mice following first passage of white-tailed deer
CWD allotypes. (A-B) Accumulation of wt-PrP^{CWD} aggregates in tg33 mice. The regional
distribution of wt-PrP^{CWD} aggregates was similar in mice receiving different CWD inocula (Fig.
3A-G). (C-E) Hippocampal degeneration (box in A) was characterized by spongiform change
and loss of pyramidal neurons of the Ammon’s horn (CA1-CA3), accompanied by extensive
accumulation of PrP^{CWD} aggregates and abundant astrocytosis (GFAP). (F-G) Cerebellum
pathology involved the loss of granular neurons and presence of prion protein deposits flanked
by astrocytes (sequential tissue sections). (H) Vacuolation in Purkinje neurons and cerebellar
white matter was observed. (I-J) Detection of S96-PrP^{CWD} aggregates in tg60 mice infected with
H95/wt and H95/S96 CWD allotypes. The distribution of PrP^{CWD} aggregates was similar
between animals receiving H95+ deer CWD inocula (Fig. 3J-K). (K) S96-PrP^{CWD} aggregates in
hippocampus were noticeable at higher magnification (small box in J). (L-M) Abnormal prion
protein deposits and spongiosis in thalamic nuclei (large box in J). Cerebellar pathology included
white matter vacuolation (N) and astrocytosis (O) that colocalized with diffuse and punctate
protein aggregates (P). (Q) Lesion profile of tg33 mice infected with deer CWD allotypes. (R)
Lesion profile of tg60 mice infected with H95+ deer CWD. Brain regions: 1 (Medulla), 2
(Cerebellum), 3 (Superior colliculus), 4 (Hypothalamus), 5 (Thalamus), 6 (Hippocampus), 7
(Septum), 8 (Posterior cortex), 9 (Anterior cortex). (A and I Bar: 2.5 mm), (B and G Bar: 1 mm),
PrP CWD detection was achieved with anti-PrP monoclonal antibody BAR224. (A: tg33 mouse infected with S96/wt CWD at 270 dpi. B: tg33 mouse infected with H95/S96 CWD at 387 dpi), (I: tg60 mouse infected with H95/S96 CWD at 375 dpi, J: tg60 mouse infected with H95/S96 CWD at 414 dpi).

Fig. 3. Distribution of PrP CWD aggregates in the brain of tg-deer-PRNP mice inoculated with different white-tailed deer CWD allotypes. (A-G). PrP CWD aggregates in the brain of tg33 mice inoculated with different white-tailed deer CWD allotypes. (H-I) Abnormal PrP aggregates were detected in the brain of non-clinical tg60 mice inoculated with wt/wt and S96/wt CWD allotypes after > 700 dpi. (J-K) Only tg60 mice inoculated with H95+ CWD allotypes had clinical signs and were consistently positive for PrP CWD aggregates. (K) Coronal brain sections from a clinically-ill tg60 mouse infected with H95+ CWD prions (414 dpi). (L-M) Mock-infected tg33 and tg60 mice. (A-D and J-K Bar: 1 mm), (E-I and M Bar: 2.5 mm), (L Bar: 5 mm). Tissue sections were stained with anti-PrP monoclonal antibody, BAR224.

Fig. 4. Disease-associated PrP-res in tg-deer-PRNP mice inoculated with different CWD allotypes. (A) PrP-res from brains of prion affected tg33 (wt-PrP C) and tg60 (S96-PrP C) mice. Brain homogenates were digested with proteinase K, analyzed by SDS-PAGE and western blot. PrP-res from tg33 mice had similar glycoform ratios (B = Quantification of PrP-res glycoform ratios in CWD infected tg33 mice) and molecular weight after enzymatic cleavage. S96-PrP-res has lower molecular weight and was detectable only in brain homogenates derived from tg60
mice infected with H95+ CWD allotypes. UI = Homogenates from tg mice inoculated with uninfected deer brain homogenate. PrP-res detection was achieved with anti-PrP monoclonal antibody BAR224.

Fig. 5. Serial passage of tg60 (S96-PrP<sup>C</sup>)-passaged CWD prions. (A) Syngeneic transmission of tg60CWD-H95<sup>+</sup> isolates into tg60 mice led to reduction in incubation period following intracranial inoculation of 10% (w/v) brain homogenates. (B) S96-PrP-res properties were maintained following secondary passage in tg60 mice. S96-PrP-res has lower molecular weight compared to wt-PrP-res derived from tg33 mice. (C-D) Distribution of S96-PrP<sup>CWD</sup> aggregates in the brain of tg60 mice infected with tg60CWD-H95<sup>+</sup> prions. Immunohistochemical comparison revealed similar distribution of abnormal PrP<sup>CWD</sup> aggregates as observed in tg60 mice from first passage of H95<sup>+</sup> deer CWD (Fig. 2I-J and 3J-K). Detection of abnormal PrP (B-D) was performed with antibody BAR224. (C-D Bar: 2.5 mm).

Fig 6. Allogeneic transmission of tg60 (S96-PrP<sup>C</sup>)-passaged CWD prions into tg33 mice. (A) Incubation periods of tg33 mice upon challenge with passage-1 tg60CWD-H95<sup>+</sup> isolates. Passage of tg60CWD-H95/S96 brain homogenate gave rise to different clinical presentations (Hyperactive vs lethargic) resembling the phenotypes described for both tg-deer-PRNP lines during the first passage of deer CWD prions. Black triangles represent tg33 animals with hyperactive disease presentation, high molecular weight PrP-res and widespread distribution of brain PrP<sup>CWD</sup> aggregates. Orange symbols correspond to tg33 mice with lethargic presentation, low molecular weight PrP-res and localized distribution of PrP<sup>CWD</sup> aggregates. (B) PrP-res
glycotypes in brain of tg33 mice inoculated with different tg60CWD-H95+ homogenates.

Infected tg33 mice accumulated different proteinase K resistant PrP types resembling those observed after first passage of deer CWD prions. (C) Divergent histological phenotypes in tg33 mice infected with different tg60CWD-H95/S96 or tg60CWD-H95/wt brain homogenate. (C Bars: 2.5 mm). Detection of abnormal PrP was performed with anti-PrP monoclonal BAR224.
Table 1. Prion disease in tg-deer-PRNP mice inoculated with white-tailed deer and tg-mice passaged CWD prions.

Table |
<table>
<thead>
<tr>
<th>Inocula</th>
<th>tg-deer-PRNP</th>
<th>CWD (Bh) Dose %</th>
<th>Positive&lt;sup&gt;a&lt;/sup&gt; /Total</th>
<th>Incubation Period- Range (dpi)</th>
<th>PrP-res Type</th>
<th>PrP&lt;sup&gt;CWD&lt;/sup&gt; Distribution Pattern</th>
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<tr>
<td>Deer CWD-wt/wt</td>
<td>tg33</td>
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<td>215 - 310 dpi</td>
<td>High MW</td>
<td>Widespread &quot;W&quot;</td>
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<td>High MW</td>
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<tr>
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<tr>
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<td>0/10</td>
<td>&gt; 600 dpi</td>
<td>Negative</td>
<td>Not Determined</td>
<td></td>
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<tr>
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<td>tg33</td>
<td>10%</td>
<td>20/20</td>
<td>258 - 329 dpi</td>
<td>High MW</td>
<td>Widespread &quot;W&quot;</td>
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<td>394 - 473 dpi</td>
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<td>331 - 369 dpi</td>
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<td>0/5</td>
<td>&gt; 560 dpi</td>
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<sup>a</sup>: Clinically positive animals.