Elk with a long incubation prion disease phenotype have a unique PrP\textsuperscript{d} profile

Katherine I. O’Rourke\textsuperscript{a}, Terry R. Spraker\textsuperscript{c}, Dongyue Zhuang\textsuperscript{a}, Justin J. Greenlee\textsuperscript{b}, Thomas E. Gidewski\textsuperscript{d} and Amir N. Hamir\textsuperscript{b}

\textsuperscript{a}United States Department of Agriculture, Agricultural Research Service, Animal Disease Research Unit, Pullman, Washington, \textsuperscript{b}Virus and Prion Disease of Livestock Research Unit, National Animal Disease Center, Ames, Iowa, \textsuperscript{c}Colorado State University and \textsuperscript{d}United States Department of Agriculture, Animal Plant Health Inspection Service, Veterinary Services, Fort Collins, Colorado, USA

Correspondence to Dr Katherine O’Rourke, USDA, ARS, ADRU, United States Department of Agriculture, Agricultural Research Service, Animal Disease Research Unit, 3003 ADBF, Pullman, WA 99164, USA
Tel: +1 509 335 6020; fax: +1 509 335 8328; e-mail: korourke@vetmed.wsu.edu

Received 30 August 2007; accepted 4 September 2007

The transmissible spongiform encephalopathies (TSEs) invariably result in fatal neurodegeneration and accumulation of PrP\textsuperscript{d}, an abnormal form of the host prion protein PrP\textsuperscript{c}, encoded by the PRNP gene. A naturally occurring polymorphism (methionine/valine) at PRNP codon 129 is associated with variation in relative disease susceptibility, incubation time, clinical presentation, neuropathology, and/or PrP\textsuperscript{d} biochemical characteristics in a range of human TSEs. A methionine/leucine polymorphism at the corresponding site in the Rocky Mountain elk PRNP gene is associated with variation in relative susceptibility and incubation time in the cervid TSE chronic wasting disease. We now report that elk lacking the predisposing 132-methionine allele develop chronic wasting disease after a long incubation period and display a novel PrP\textsuperscript{d} folding pattern. NeuroReport 18:1935–1938 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins

Keywords: cervid, chronic wasting disease, Creutzfeldt–Jakob disease, glycoform profile, prion protein, Rocky Mountain elk, Western blot

Introduction

The transmissible spongiform encephalopathies (TSEs) are a heterogeneous group of fatal neurologic disorders that include the livestock diseases like sheep scrapie, bovine spongiform encephalopathy (BSE), and cervid chronic wasting disease (CWD), as well as a spectrum of sporadic, iatrogenic, familial, and acquired human TSEs (reviewed in Ref. [1]). Expression of the cellular prion glycoprotein PrP\textsuperscript{c}, encoded by the mammalian prion protein precursor gene PRNP, is necessary for the development of TSEs [2]. PrP\textsuperscript{c} is ubiquitously expressed in mammalian tissues and is converted to the disease-associated, partially protease resistant isoform PrP\textsuperscript{d} in a limited number of cell types through a series of posttranslational changes. The abnormal prion protein is the major component of the transmissible agent, the proteinaceous infectious particle or prion [3]. In infectious TSEs, PrP\textsuperscript{c} is converted to PrP\textsuperscript{d} following uptake of exogenous prions through the food supply or through accidental parenteral exposure to contaminated blood, tissues, or surgical instruments. Misfolding occurs spontaneously in individuals with pathogenic mutations in the PRNP gene and sporadically in aged individuals with the wild type allele. In humans, a polymorphism at PRNP codon 129 (methionine, M to valine, V) is associated with relative susceptibility to sporadic and iatrogenic forms of the TSE Creutzfeldt–Jakob disease (CJD) and modulates the disease phenotype in certain familial TSEs [1]. Variant CJD (vCJD) and kuru are human TSEs with an infectious etiology, related to oral exposure to bovine and human prions, respectively. To date, all reported cases of vCJD originating from exposure to bovine prions have occurred in individuals homozygous for the allele encoding 129M, although the 129V allele is not protective following inadvertent exposure to prions in transfused blood [4]. Similarly, homozygosity for 129M predisposes exposed individuals to kuru and homozygosity for either allele is associated with earlier mean age of onset. As with vCJD, homozygosity is not protective [5].

Cervus elaphus nelsoni (the Rocky Mountain elk) is the only other mammalian species with a naturally occurring TSE [6] and a polymorphism (M or leucine, L) at the corresponding site [7], numbered codon 132 in cervid ruminants because of upstream insertions. Homozygosity for 132M predisposes elk to CWD following natural challenge [8] and heterozygosity was associated with a near doubling of incubation time following experimental oral challenge [9]. We now report that elk homozygous for 132L developed clinical signs of CWD at nearly triple the incubation interval of 132MM elk, in spite of accumulation of PrP\textsuperscript{d} in the lymphoreticular system midway during the preclinical stage of disease and detectable in postmortem retropharyngeal lymph nodes collected after prolonged incubation times. Homozygosity for 132L was associated with a novel folding pattern resulting in a shift of the proteinase K cleavage site in samples from 132LL elk when compared with 132MM or 132LM elk with CWD.
Methods

All work was conducted under the guidelines of the Institutional Animal Care and Use Committee at the National Veterinary Services Laboratory, Ames, Iowa. The source, genotyping, husbandry, and oral inoculation of the elk in this study have been described [9]. Briefly, elk homozygous for 132L were sourced from a farm in which CWD was diagnosed in 79 elk with the 132MM and 132LM genotypes. No cases were found in 132LL elk. 132LL (n=4), 132MM (n=2), and 132LM (n=2) elk were each inoculated by the oral route using 15 g of pooled brain (equal parts 132MM and 132LM donor tissue). Clinical signs were observed during month 23 postinoculation in both 132MM elk and at 40 months postinoculation in both 132LM elk. 132LL elk were euthanized in late-stage clinical disease (inability to rise from sternal recumbency, n=2), early clinical disease (mild weight loss, rough hair coat, behavioral changes observable only to experienced caretakers, n=1), or preclinically (n=1). CWD was confirmed by histology and immunohistochemistry analysis of the medulla at the level of the obex as described [10]. Peripheral distribution of PrPd was defined as PrPd in retropharyngeal lymph nodes level of the obex as described [10].

Results

Prolonged and variable incubation time with peripheral PrPd accumulation in leucine homozygous elk

DNA sequence analysis of the PRNP open reading frame in samples from the four orally challenged elk in this study demonstrated a single nonsynonymous change at codon 132 (atg=M to ttg=L) with no other polymorphisms. Clinical disease was observed during month 59 in one elk and during month 63 in a second animal. The two remaining 132LL elk were euthanized during month 64 postinfection, at which time one elk was displaying early signs of clinical disease, primarily subtle behavioral changes, slight weight loss, and roughened hair coat. The fourth elk was clinically normal at the time of euthanasia. Three of the 132LL elk had detectable PrPd in antemortem biopsy samples of rectal mucosal lymphoid tissue and all four 132LL elk had detectable PrPd in retropharyngeal lymph node collected at necropsy.

Immunoblot analysis of disease-associated prion protein in leucine homozygotes is characterized by a shift in the prion protein K cleavage site

Detergent extracts of brain were examined by Western blot analysis using a panel of monoclonal antibodies. The three characteristic bands of the proteinase-resistant core of PrPd, presumably representing the diglycosylated, monoglycosylated, and unglycosylated forms, were observed in all immunoblots probed with mAb 99/97.6.1. The band with the lowest apparent molecular weight comigrated with fully deglycosylated samples (Fig. 1a) in samples from all elk and represents the proteinase K resistant core of PrPd in the samples. The mean apparent molecular mass of this band in samples from the four 132LL elk was approximately 18.5 kDa, significantly lower than the mean mass of corresponding bands from the 132MM (19.97) or 132LM (20.13) elk. Representative data are shown in Fig. 1a and c; mean values (±SD) of two to four replicate blots for each of the four 132LL elk and the control 132MM and 132LM elk are shown in Fig. 2. Epitope mapping with a panel of monoclonal antibodies demonstrated that the reduction in molecular mass in samples from 132LL elk was associated with the loss of the amino terminal epitope bound by mAb 8G8 (Fig. 1b). The weak signal observed with antibody 8G8 (Fig. 1c) indicates that the cleavage occurred near residues 98–113, rather than at the predicted cleavage site at residue 78 or 82 [15].

![Fig 1](image_url) Comparative Western blot analysis of proteinase K resistant PrPd bands from Rocky Mountain elk with genotypes 132MM (lanes 1, 2), 132LL (lanes 3, 4), and 132LM (lanes 5, 6) detected with (a) mAb 99/97.6.1, (b) p4, or (c) 8G8. (a) Samples were examined with (+) or without (−) peptide-N-glycosidase enzymatic deglycosylation. Arrows indicate molecular mass markers (40 000 and 30 000).
polymorphism at PRNP molecular biology associated with this genotype. The [4] and no further data are available on pathogenesis or unrelated cause; PrPd was found in lymphoreticular tissues a heterozygote was an elderly patient who died from an from infected, preclinical donors. The single case reported in receiving transfusions of nonleuko-depleted red blood cells who developed clinical signs of disease 6–8.5 years after human blood products were methionine homozygotes [17].

Three of the four cases of vCJD associated with exposure to prion-contaminated human blood products were methionine homozygotes. Three of the four cases of vCJD associated with exposure to prion-contaminated human blood products were methionine homozygotes [17] who developed clinical signs of disease 6–8.5 years after receiving transfusions of nonleuko-depleted red blood cells from infected, preclinical donors. The single case reported in a heterozygote was an elderly patient who died from an unrelated cause; PrPd was found in lymphoreticular tissues [4] and no further data are available on pathogenesis or molecular biology associated with this genotype. The polymorphism at PRNP 129, however, is not sufficient to account for the observed heterogeneity in the human TSEs. Although differences in assay methodology among laboratories have resulted in some inconsistencies in molecular characterization, at least two molecular patterns associated with a shift in the proteinase K cleavage site are recognized in methionine homozygotes with sporadic CJD. The pattern generally considered type 1 is observed only in methionine homozygotes. The type 2 pattern is observed in all genotypes, and differences in clinical presentation are associated with genotype in the absence of molecular variation detected by Western blot [18,19]. Both types 1 and 2 in methionine homozygotes are clearly distinguishable from the type 4 pattern observed in vCJD, which is characterized by a lower molecular weight in the unglycosylated band [20].

Molecular typing of ruminant TSE samples is less well defined. BSE samples collected during the height of the outbreak were relatively uniform, with the lowest band migrating faster than the corresponding band from sheep with scrapie, allowing Western blot discrimination between BSE and sheep scrapie strains using molecular mass and the discriminatory antibody P4 [13,21]. Intensive surveillance for BSE has now revealed samples with a prion protein migration pattern higher (H type) or lower (L type) than the classical samples (C type) [22–24]. The well characterized experimental ovine scrapie strains SSBP/1 and CH1641 differ in PRNP genotype and molecular conformation [21], although molecular correlates for the more than 20 strains of ovine scrapie identified in mice are lacking. CH1641 shares some biochemical characteristics with the 132LL samples examined in this study, notably the loss of the epitope for mAb P4 and conservation of reduced but detectable immunoreactivity with mAb 8G8.

In this study of Rocky Mountain elk, incubation time and immunoblot patterns from leucine homozygotes were distinctly different from those of the 132M homozygotes. Although heterozygous elk had an incubation time intermediate between the two homozygous groups, immunoblots of samples from the heterozygous elk were similar to those from the 132M homozygotes and there was no evidence of a hybrid Western blot pattern. The 132M-derived PrPd may have been sufficient, even at the half dose expected in heterozygotes, to cause fatal neurodegeneration in 132LM elk before the more slowly misfolding 132L PrPd could be detected.

The mechanisms for prolonged incubation times are not known. A long incubation phenotype is observed in natural infectious scrapie in sheep heterozygous for the alleles encoding V at codon 136 and arginine at codon 171. In sheep of this genotype, PrPd accumulates in the central nervous system but is not detected in the lymphoreticular system, suggesting that the lag in clinical onset may be due to the lack of a peripheral prion amplification site. In contrast, all elk in the study had detectable levels of PrPd in the retropharyngeal lymph node, the most reliable indicator of peripheral accumulation in elk [10] and three of the 4 132LL elk had detectable levels of PrPd at 49 months postinfection in rectal mucosal lymphoid tissue, a site that is accessible for antemortem sampling but has an estimated sensitivity of 85% in preclinically infected elk with CWD [11] and sheep with scrapie [25]. Serial sampling of infected elk throughout the incubation period and examination of neural and lymphoid subsets in which PrPd accumulates will be helpful in defining the host-specific factors controlling incubation time. The genotype of the donor tissue may also have contributed to the prolonged incubation time. Incubation time is related to prion titer in experimental systems. Donor tissue was prepared from one 132MM and one 132LM elk with clinical CWD. As with the experimental samples, no evidence of truncated PrPd, presumably of 132L origin, was observed in the donor tissue. If conversion of 132L PrPd is inefficient when exposed to 132M PrPd, the low titer of 132L PrPd in the inoculum may have resulted in the prolonged incubation time. Experimental challenge of elk of each genotype using brain homogenates from 132LL elk should be helpful in evaluating the relative contribution of donor and recipient genotypes on incubation time.

**Discussion**

The interplay of genotype, strain, disease characteristics, and prion protein molecular biology in the human TSEs is complex. In humans exposed to kuru, heterozygosity at codon 129 is associated with the lowest rates of infection. Methionine homozygotes are predisposed to disease [16], with shorter incubation times than those observed in valine homozygotes or in heterozygotes [5]. To date, cases of vCJD have been methionine homozygotes. Three of the four cases of vCJD associated with exposure to prion-contaminated human blood products were methionine homozygotes [17] who developed clinical signs of disease 6–8.5 years after receiving transfusions of nonleuko-depleted red blood cells from infected, preclinical donors. The single case reported in a heterozygote was an elderly patient who died from an unrelated cause; PrPd was found in lymphoreticular tissues [4] and no further data are available on pathogenesis or molecular biology associated with this genotype. The polymorphism at PRNP 129, however, is not sufficient to account for the observed heterogeneity in the human TSEs. Although differences in assay methodology among laboratories have resulted in some inconsistencies in molecular characterization, at least two molecular patterns associated with a shift in the proteinase K cleavage site are recognized in methionine homozygotes with sporadic CJD. The pattern generally considered type 1 is observed only in methionine homozygotes. The type 2 pattern is observed in all genotypes, and differences in clinical presentation are associated with genotype in the absence of molecular variation detected by Western blot [18,19]. Both types 1 and 2 in methionine homozygotes are clearly distinguishable from the type 4 pattern observed in vCJD, which is characterized by a lower molecular weight in the unglycosylated band [20].

Molecular typing of ruminant TSE samples is less well defined. BSE samples collected during the height of the outbreak were relatively uniform, with the lowest band migrating faster than the corresponding band from sheep with scrapie, allowing Western blot discrimination between BSE and sheep scrapie strains using molecular mass and the discrimination of antibody P4 [13,21]. Intensive surveillance for BSE has now revealed samples with a prion protein migration pattern higher (H type) or lower (L type) than the classical samples (C type) [22–24]. The well characterized experimental ovine scrapie strains SSBP/1 and CH1641 differ in PRNP genotype and molecular conformation [21], although molecular correlates for the more than 20 strains of ovine scrapie identified in mice are lacking. CH1641 shares some biochemical characteristics with the 132LL samples examined in this study, notably the loss of the epitope for mAb P4 and conservation of reduced but detectable immunoreactivity with mAb 8G8.

In this study of Rocky Mountain elk, incubation time and immunoblot patterns from leucine homozygotes were distinctly different from those of the 132M homozygotes. Although heterozygous elk had an incubation time intermediate between the two homozygous groups, immunoblots of samples from the heterozygous elk were similar to those from the 132M homozygotes and there was no evidence of a hybrid Western blot pattern. The 132M-derived PrPd may have been sufficient, even at the half dose expected in heterozygotes, to cause fatal neurodegeneration in 132LM elk before the more slowly misfolding 132L PrPd could be detected.

The mechanisms for prolonged incubation times are not known. A long incubation phenotype is observed in natural infectious scrapie in sheep heterozygous for the alleles encoding V at codon 136 and arginine at codon 171. In sheep of this genotype, PrPd accumulates in the central nervous system but is not detected in the lymphoreticular system, suggesting that the lag in clinical onset may be due to the lack of a peripheral prion amplification site. In contrast, all elk in the study had detectable levels of PrPd in the retropharyngeal lymph node, the most reliable indicator of peripheral accumulation in elk [10] and three of the 4 132LL elk had detectable levels of PrPd at 49 months postinfection in rectal mucosal lymphoid tissue, a site that is accessible for antemortem sampling but has an estimated sensitivity of 85% in preclinically infected elk with CWD [11] and sheep with scrapie [25]. Serial sampling of infected elk throughout the incubation period and examination of neural and lymphoid subsets in which PrPd accumulates will be helpful in defining the host-specific factors controlling incubation time. The genotype of the donor tissue may also have contributed to the prolonged incubation time. Incubation time is related to prion titer in experimental systems. Donor tissue was prepared from one 132MM and one 132LM elk with clinical CWD. As with the experimental samples, no evidence of truncated PrPd, presumably of 132L origin, was observed in the donor tissue. If conversion of 132L PrPd is inefficient when exposed to 132M PrPd, the low titer of 132L PrPd in the inoculum may have resulted in the prolonged incubation time. Experimental challenge of elk of each genotype using brain homogenates from 132LL elk should be helpful in evaluating the relative contribution of donor and recipient genotypes on incubation time.

**Conclusion**

The prion disorders are characterized by long incubation times, even in individuals with predisposing genotypes. In the absence of a reliable blood test, an extremely long incubation period in a subset of infected carriers represents a major hurdle for disease control if peripheral PrPd
accumulation is associated with persistent shedding of the agent. Rocky Mountain elk thus provide a useful model for examining a range of pathogenic mechanisms associated with a PRNP site of critical importance in both vCJD and CWD.

Acknowledgements
This project was funded by Grant 5348-32000-021-00D from the US Department of Agriculture. The authors are grateful for the excellent care provided to the animals by USDA APHIS and ARS personnel. L. Creekmore, USDA APHIS Veterinary Services, participated in the initiation of this experiment and K. VerCauteren, USDA APHIS Wildlife Services, assisted with live animal sampling. The Elk Research Council of the North American Elk Breeders Association and Drs T. Cline and S. Holland of the South Dakota Animal Industry Board provided assistance in identification and acquisition of donor and recipient elk. No conflicts of interest exist.

References