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Differential immunoreactivity of goat derived scrapie following *in vitro* misfolding versus mouse bioassay

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ABSTRACT

The protein misfolding cyclic amplification (PMCA) assay allows for detection of prion protein misfolding activity in tissues and fluids from sheep with scrapie where it was previously undetected by conventional western blot and immunohistochemistry assays. Studies of goats with scrapie have yet to take advantage of PMCA, which could aid in discerning the risk of transmission between goats and goats to sheep. The aim of the current study was to adapt PMCA for evaluation of scrapie derived from goats. Diluted brain homogenate from scrapie-infected goats (i.e., the scrapie seed, PrP^{Sc}) was subjected to PMCA using normal brain homogenate from ovinized transgenic mice (*tg338*) as the source of normal cellular prion protein (the substrate, PrP^{C}). The assay end-point was detection of the proteinase K-resistant misfolded prion protein core (PrP^{res}) by western blot. Protein misfolding activity was consistently observed in caprine brain homogenate diluted 10,000-fold after 5 PMCA rounds. Epitope mapping by western blot analyses demonstrated that PrP^{res} post-PMCA was readily detected with an N-terminus anti-PrP monoclonal antibody (P4), similar to scrapie inoculum from goats. This was in contrast to limited detection of PrP^{res} with P4 following mouse bioassay. The inverse was observed with a monoclonal antibody to the C-terminus (F99/97.6.1). Thus, brain homogenate prepared from uninoculated *tg338* served as an appropriate substrate for serial PMCA of PrP^{Sc} derived from goats. These observations suggest that concurrent PMCA and bioassay with *tg338* could improve characterization of goat derived scrapie.

1. Introduction

Scrapie is a prion disease of sheep and goats that is part of a larger group of transmissible spongiform encephalopathies including bovine spongiform encephalopathy (BSE), chronic wasting disease in cervids, transmissible mink encephalopathy, and variant Creutzfeldt–Jakob disease in humans. This neurodegenerative disorder is characterized by the conversion of normal cellular prion protein (PrP^{C}) to a misfolded conformation (PrP^{Sc}), which includes a protease resistant core peptide (PrP^{res}) [1,2]. Antemortem diagnosis relies on detection of PrP^{Sc} in peripheral lymphoid tissues, e.g. rectoanal mucosa-associated lymphoid tissue [3,4]. Transmission of scrapie in sheep may occur via exposure to PrP^{Sc} that is present in the placenta [5,6], blood [7,8], and milk [9,10] from infected ewes with similar routes of transmission anticipated for goats [11,12]. Despite assumed disease transmission through a variety of biological sources, detection of PrP^{Sc} in fluids and some tissues by conventional western blot and immunohistochemistry methods can be challenging.

Protein misfolding cyclic amplification (PMCA) is an *in vitro* process thought to mimic the *in vivo* conversion of PrP^{C} to PrP^{res}. PMCA is generally conducted in repeating cycles of seeded conversion (incubation phase) followed by disaggregation (sonication phase) [13,14]. Not only has PMCA been utilized to amplify very dilute PrP^{Sc} seed [15,16], but newly formed PrP^{Sc} can be infectious [16,17] and maintain strain-specific properties [18]. To date, PMCA has proven to be a useful tool in detecting PrP^{Sc} from several species [19–25] including humans [26] and sheep [20,27].

Through PMCA, scrapie associated prion misfolding activity has been confirmed in tissues from sheep in which PrP^{Sc} detection by conventional immunoassays is limited. Such samples include blood [27], milk [28], saliva [29], feces [30], and urine [31]. Detection of PrP^{Sc} in goats by conventional immunoassays appears to be, at least in some tissues, more limited than sheep. However, PMCA, which could aid in investigating the transmission risk from goat to goat and goat to sheep, has been minimally applied to caprine tissues [20]. The amount of available PrP^{C} is a critical factor in

Abbreviations: gNBH, goat normal brain homogenate; mNBH, mouse normal brain homogenate; mAb, monoclonal antibody; PK, proteinase K.

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assay sensitivity with evidence suggesting that the greater concentration of PrP^C expressed in the brain of transgenic mice improves amplification of PrP^{Sc} [21,32,33]. Thus, the aim of the current study was to evaluate PrP^{Sc} from goats amplified by PMCA using normal brain homogenate from ovinized mice as the source of PrP^C and compare it to misfolded prion generated *in vivo* by mouse bioassay using the same ovinized mouse strain.

2. Materials and methods

2.1. Animals, tissue collection, homogenate preparation

All animal handling and tissue collection were performed according to protocols approved prior to study initiation by the Washington State University and University of Washington Institutional Animal Care and Use Committees. One Nubian goat (case number 3953) and two domestic mixed breed goats (3538 and 30-75) were identified as infected with naturally acquired scrapie. The Nubian goat and two mixed breed goats were acquired from different flocks. Postmortem disease confirmation was performed at the National Veterinary Services Laboratory, USDA, Ames IA, USA, using immunohistochemistry analysis of brainstem with monoclonal antibody (mAb) F99/97.6.1. At the time of death, brain tissue was harvested and stored at −80 °C. Homogenates (10% w/v) were prepared in sterile saline as described [34] and stored at −80 °C until bioassay or PMCA. The presence of PrP^{Sc} was confirmed by western blot analysis of PrP^{res}.

Brain tissue was also harvested from a scrapie-free Saanen goat (4111; Washington State University flock) at euthanasia and stored at −80 °C. No PrP^{Sc} was detected by immunohistochemistry analyses of brainstem and retropharyngeal lymph node. Medulla and pons of the hindbrain were homogenized with a disposable tissue grinder (VWR) in ice–cold conversion buffer [PBS supplemented with 150 mM NaCl, 4 mM EDTA, 1% Triton X-100, and complete protease inhibitor (Roche)] to yield a 10% (w/v) normal brain homogenate (gNBH). Homogenates were centrifuged at 500*g* for 60 s at 4 °C to remove large particulate matter and aliquots stored at −80 °C.

Scrapie-free ovinized mice (strain *tg338*, [35,36]) provided brain tissue for PMCA substrate. Breeding pairs of transgenic *tg338* mice were kindly provided by Dr. Hubert Laude (Institut National de la Recherche Agronomique, France) and held in a breeding colony at the University of Washington (UW). The presence of the transgene was confirmed by DNA sequence analysis of tail snips. Whole brains were harvested at the time of euthanasia and stored at −80 °C until homogenization in conversion buffer as described above to create 10% normal brain homogenate (mNBH) for PMCA.

2.2. Serial protein misfolding cyclic amplification

Serial PMCA was performed as previously described [15,27,37] with the following modifications. Caprine brain homogenate (10% = 10^{-1}) was thawed on ice and diluted 10-fold into conversion buffer. The resulting 1% homogenate (10^{-2}) was further diluted 10-fold into NBH to yield pre-amplification dilutions of 10^{-3} to 10^{-10} of which 100 μl each were transferred to 0.2 ml reaction tubes. The remaining volume of each dilution was stored at −80 °C (unamplified control). Reaction tubes were placed in a microplate horn sonicator (Misonix S-4000; Qsonica) containing water maintained at 37 °C by a recirculating chiller (VWR) and incubated 1 hour prior to the first sonication. Samples underwent cycles of sonication (ultrasonic amplitude of 75 for 40 s) and incubation (59 min 20 s). At the end of 48 cycles, defined as one round, samples were diluted 1:3 into fresh NBH before initiating the next round of PMCA; remaining product was stored at −80 °C. Up to five rounds of PMCA were performed.

2.3. Bioassay

Bioassay in transgenic ovinized mice (*tg338*) was recently described by O'Rourke and colleagues [34]. Inoculated animals and age-matched uninoculated controls were monitored daily for appearance of clinical signs and culled at terminal disease. Brains were collected at euthanasia and frozen at −20 °C.

2.4. Western blot

Western blot analyses of PMCA products and brain homogenates from goats or inoculated *tg338* mice were performed as described elsewhere [34] except that all samples were incubated with 200 μg/ml proteinase K (PK) at 37 °C for 90 min prior to electrophoresis. Immunodetection of PrP^{res} was performed with either primary mAb F99/97.6.1 (3.5 μg/ml; VMRD Inc.) or mAb P4 (0.2 μg/ml; R-Biopharm AG) and HRP-conjugated goat anti-mouse IgG_1 (1:5000; Southern Biotechnology) or HRP-conjugated goat anti-mouse IgG, H + L (1:7000; KPL). The chemiluminescent signal (Amersham ECL; GE Healthcare) was captured on film (Kodak BioMax); digital images were obtained with an Alpha Innotec image analyzer (Alpha Innotech Corp.).

2.5. Proteinase K and epitope site mapping

Polymerase chain reaction and DNA sequence analysis of the open reading frame by standard methods was used to determine diploid *PRNP* genotypes of donor goats [38] and ovinized mice [39]. The open reading frames were translated and aligned using Vector NTI Advance software (version 11.5.1; Invitrogen). Putative PK cleavage sites were determined using the online resource "Peptide cutter" (http://web.expasy.org/peptide_cutter/). The published PrP epitope sequences recognized by mAbs P4 [40] and F99/97.6.1 [41] were used for alignment.

3. Results and discussion

3.1. Serial PMCA of PrP^{Sc} from goats

Selection of substrate is an important component in amplification of PrP^{Sc} by PMCA. Early studies with PMCA utilized brain homogenate prepared from uninfected individuals of the same species as the misfolded seed [20]. Thus, our initial PMCA reactions utilized homogenate prepared from medulla and hindbrain of an uninfected goat (gNBH). Interestingly, this substrate did not support protein misfolding regardless of various combinations of sonication time/power, incubation, and number of cycles per round (data not shown). Recent reports demonstrated that ovinized transgenic mice, *tg338*, can be a source of substrate for PMCA analyses of ovine scrapie [32,33] and *tg338* has also been successfully used for bioassay of caprine scrapie [34]. Thus, we selected brain from healthy, uninoculated *tg338* to serve as PMCA substrate. *Tg338* brain homogenate (mNBH) prepared in PMCA conversion buffer supported misfolding of PrP^{Sc} from diluted caprine brain (Fig. 1). A 100-fold or greater dilution of 10% caprine brain homogenate resulted in weak to no western blot detection in samples pre-PMCA. After one PMCA round, the pre-PMCA dilution of 10^{-3} was more readily detected by western blot with the signal becoming stronger after each additional round. Pre-PMCA dilutions of 10^{-4} and 10^{-5} were consistently detected by rounds 3 and 5, respectively, in all samples tested. Reactions were terminated at round 5 as a low level of spontaneous misfolding was detected in the negative control (10^{-3} gNBH in mNBH).

Several factors may have contributed to this. First, PrP^C concentration is critical to optimal amplification [21] and *tg338* is an

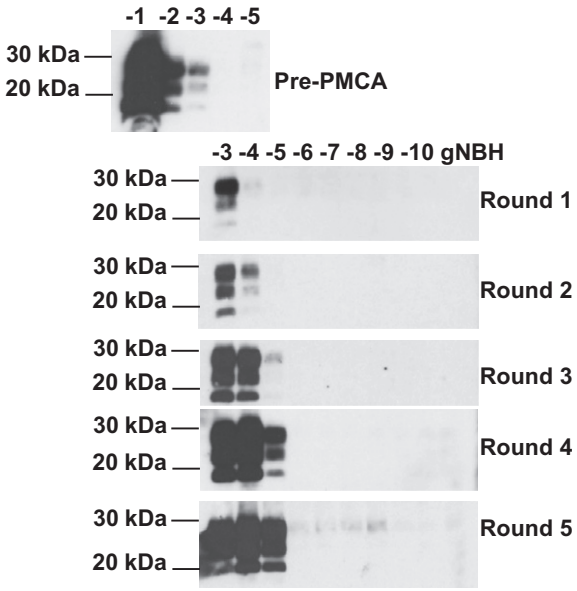

Fig. 1. Amplification of PrP^{Sc} from caprine brain by serial PMCA with uninfected *tg338* mouse brain as substrate. Caprine brain homogenate (550 μg starting wet weight; 10% = 10^{-1}, represented as −1) was serially diluted into mNBH (10^{-2}–10^{-10}; represented as −2 to −10) and subjected to five PMCA rounds of 48 cycles of sonication and incubation. Samples were diluted 1:3 into fresh mNBH between rounds. Brain from an uninfected goat (gNBH) was diluted to 10^{-3} in mNBH as a PMCA negative control. Pre- and post-PMCA samples underwent PK digestion (200 μg/ml for 90 min at 37 °C) prior to western blot analysis with mAb P4. Shown are representative blots from one of three goats tested (animal 3953). Molecular mass markers are indicated on the left.

over-expresser of ovine PrP^{C} [35,36] (Supplementary Fig. S1). Further, whole mouse brains were used to prepare mNBH whereas caprine medulla and hindbrain were selected for preparation of gNBH as this is a major site of PrP^{Sc} accumulation in classical ovine and caprine scrapie [42]. It is possible that factors present in other parts of the brain that were present in the mNBH preparation influenced misfolding. Finally, an additional factor that may have contributed to a lack of detectable amplification with gNBH may be the *PRNP* genotype of the donor tissue. Evidence suggests that in sheep the genotype of the substrate donor contributes to PMCA efficiency [27]. In this study, mismatches in the *PRNP* open reading frame between the normal brain homogenates from goat 4111 or *tg338* mice and the tissues from infected goats (3953, 3538, and 30-75) were observed at translated codons 127 and 240 (Supplementary Fig. S2). While it is possible that this may have compromised misfolding by PMCA with gNBH, it is not likely as we observed amplified misfolding with all three scrapie seeds with mNBH despite variation between seed and substrate. We also compared translated amino acid sequences of the goats with scrapie to mNBH at codons 136, 154, and 171 as these sites putatively impact PMCA when assessing ovine scrapie [27,43]. The *tg338* mouse is homozygous for valine at codon 136, arginine (R) at codon 154, and glutamine (Q) at codon 171; all goats used in this study are homozygous for alanine at codon 136 (136AA), 154RR, 171QQ. Unlike previous observations [27], the difference at codon 136 between the PMCA seed and substrate did not appear inhibit amplification of PrP^{Sc}. Together, our observations suggest that overexpression of PrP^{C} with the highly convertible 136 V allele in *tg338* brain yields a more efficient substrate than caprine brain for PMCA of goat derived scrapie.

3.2. Differential immunoreactivity following PMCA versus bioassay

Given the successful amplification of goat-derived PrP^{Sc} by serial PMCA when using mNBH and our previous success in transmitting scrapie infection in *tg338* mouse bioassay [34], we examined possible variations in immunoreactivity of misfolded prion amplified by serial PMCA versus that accumulating during bioassay. First, protein loading within sample type was maintained and immunodetection performed using monoclonal antibodies to epitopes located in the N- and C-terminus of the full-length prion protein. Analysis using the N-terminal mAb P4 demonstrated strong PrP^{res} labeling in the seed/inoculum and PMCA product but limited labeling of PrP^{res} from mouse brain post-primary passage bioassay (Fig. 2A). This was consistent with a previous observation by O'Rourke and colleagues [34]. In contrast, analysis using the C-terminal mAb F99/97.6.1 demonstrated relatively weak labeling of PrP^{res} in the seed/inoculum and PMCA product but strong labeling post-bioassay, i.e., the reciprocal result as compared to mAb P4 labeling. Next, western blots were prepared by adjusting sample loading to enhance PrP^{res} visualization with each monoclonal antibody (Fig. 2B). In order to more adequately visualize PrP^{Sc} with F99/97.6.1, approximately two times more seed/inoculum and PMCA product were loaded per lane. This is in contrast to less than half as much sample needed for detection with F99/97.6.1 after mouse bioassay when compared to the amount loaded for P4 detection. Thus, availability of P4 and F99/97.6.1 epitopes varied following *in vitro* versus *in vivo* protein misfolding.

The strong signal with mAb P4 in caprine brain homogenate and post-PMCA versus the weak signal post-bioassay and the reciprocal response with F99/97.6.1 were unexpected in light of previous observations of PrP^{Sc} from scrapie-infected goats passaged in *tg338* [34]. In that study, the electrophoretic profile of PrP^{res} differed from the predominant profile observed in a sample of naturally infected goats and was hypothesized to result from a loss of the P4 epitope. The detection with P4 following primary passage shown here is more similar to observations of primary passage of classical ovine scrapie in *tg338* [44] whereas the further reduction of signal upon secondary passage agrees with P4 epitope loss upon sub-passage of goat-derived scrapie isolates in *tg338* [34].

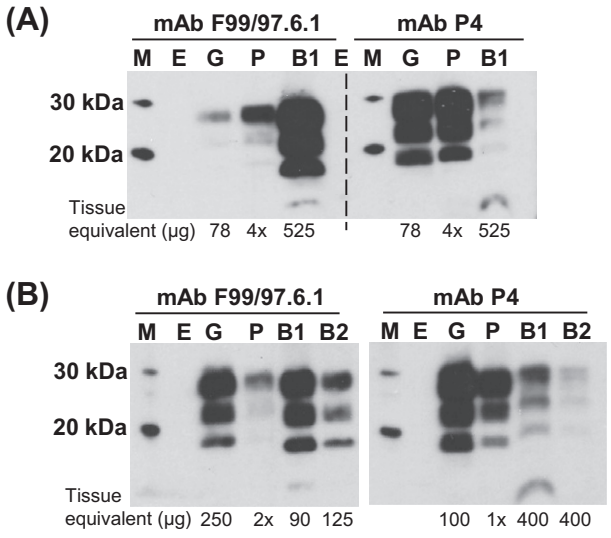

Fig. 2. Variable immunodetection of caprine brain homogenate, serial PMCA, and bioassay products with monoclonal antibodies directed to N-terminal and C-terminal epitopes of PrP. Representative western blots are shown for 3953 scrapie seed/inoculum (G), PMCA product (P), and bioassay products (B1 = passage 1, B2 = passage 2) after PK digestion (200 μg/ml for 90 minutes at 37 °C). In panel A, all samples were loaded on the same gel for electrophoresis and transfer to PVDF. Prior to immunodetection with mAb F99/97.6.1 (C-terminus) or mAb P4 (N-terminus), the membrane was cut (vertical dashed line). In panel B, loading was adjusted for optimal visualization with respective monoclonal antibodies; samples were run on two separate gels. M = molecular mass marker; E = empty lane; tissue equivalents in μg are listed at the base of each lane except for P where 1X = 1 μl PMCA product after PK digestion.

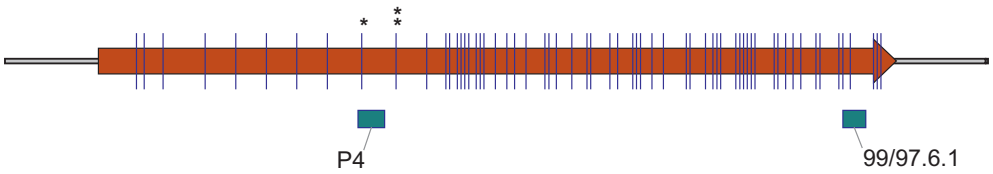

Fig. 3. Proteinase K cleavage sites and monoclonal antibody epitopes on PrP^C expressed in ovinized mouse. Positions of the putative PK cleavage sites (vertical lines) and anti-PrP epitopes (solid rectangles) recognized by mAbs P4 and F99/97.6.1 along the mature peptide (solid arrow) of ovine PrP as expressed in *tg338* mice. Putative cleavage sites immediately N- and C-terminal to P4 indicated by $*$.

The varied epitope availability between protein misfolded *in vitro* (PMCA) and *in vivo* (bioassay) suggests the presence of two PK-resistant species that are of similar molecular mass in the original tissue preparation from the goats utilized in this study. We hypothesize that one species is preferentially misfolded by PMCA and maintains the P4 epitope. The other species is preferentially misfolded by bioassay and is readily detected by F99/97.6.1. It is possible that reduction of the P4 epitope between inoculum, primary, and secondary passage results from biologic adaptation of goat derived scrapie to the mouse host despite sole expression of the ovine *PRNP* transgene. As illustrated in Fig. 3, there are putative PK sites located on either side of the P4 epitope of mature ovine PrP. Promiscuity at the PK site N-terminal (indicated by $*$) to the P4 epitope resulting in partial removal of the epitope sequence or complete loss due to cleavage at the site directly C-terminal (stacked $*$) to the P4 epitope may contribute to signal variation as the resulting mixture of PrP^{Sc} would include proteins with the complete and incomplete or missing epitope sequences. It is also possible that variation in detection of PrP^{Sc} with the P4 antibody could be a result of multiple strains more readily distinguished upon serial passage in *tg338* mice [45] but no indication of multiple strains was suggested for these inocula in observations by O'Rourke and colleagues [34]. In contrast to our hypothesis of two highly similar species present in the inoculum is that there is a single PK-resistant species that responds differently to *in vitro* and *in vivo* misfolding methods resulting in PK cleavage sites near the C-terminus more readily available post-PMCA whereas cleavage sites in the N-terminus are more readily available post-bioassay. Biological factors that are present and active *in vivo* (e.g. proteases) putatively influence folding. While these may be present in the brain homogenate used as substrate, they are potentially inactive due to chemicals included in the conversion buffer (protease inhibitors, EDTA, and triton X-100) allowing for alternative misfolding following PMCA versus that observed post-bioassay. Additional studies are needed to examine these possibilities.

3.3. Summary

The transgenic ovinized mouse, *tg338*, is a valuable tool for bioassay and PMCA analyses of ovine scrapie in regards to strain characterization [44–46] and assessment of protein misfolding activity in tissues and fluids that do not demonstrate detectable PrP^{Sc} by conventional immunoassay [32,33]. This study provides the first description of *tg338* brain as substrate for *in vitro* studies of protein misfolding of goat derived scrapie by serial PMCA, extending our previous observation that *tg338* is a valuable tool for analysis of caprine scrapie by bioassay [34]. In addition, it appears that methodology (*in vitro* versus *in vivo* misfolding) may impact characterization when molecular characteristics, such as epitope mapping, are critical to defining scrapie strains. Assessment of scrapie from goats by serial PMCA using *tg338* mNBH may provide benefits to traditional mouse bioassay in that results are more rapidly achieved and putatively yield PrP^{Sc} with qualities associated with the P4 epitope more similar to that occurring in the natural host. Studies of mouse-adapted and human strains of prion disease have demonstrated that strain characteristics such as electrophoretic mobility, glycoform analysis, and PK sensitivity are maintained by PMCA [18]. Future studies will begin to address if concurrent PMCA and bioassay with *tg338* improves characterization of caprine, and possibly ovine, scrapie strains. In addition, the methodologies described here-in will be adapted for the assessment of other caprine tissues and fluids as potential sources of scrapie transmission to goats and sheep.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.034.

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