SHORT COMMUNICATION

Stability of Bovine Spongiform Encephalopathy Prions: Absence of Prion Protein Degradation by Bovine Gut Microbiota

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Impacts

- Effects of anaerobic fermentation processes in cattle on the stability of bovine spongiform encephalopathy (BSE)-associated prion protein (PrP^{Sc}) are still unresolved.
- Study was performed to assess the ability of complex ruminal and colonic microbiota of cattle to degrade BSE-associated PrP^{Sc} in order to gain more information about the fate of PrP^{Sc} during polygastric digestion.
- No significant decrease in PrP^{Sc} levels in BSE brain homogenates was detected after co-incubation with intestinal fluids. These results indicate that BSE-associated PrP^{Sc} survive gastrointestinal digestion processes in cattle and might be excreted via faeces.

Keywords:
Bovine spongiform encephalopathy; cattle; prion protein; degradation; gut; excretion

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Summary

Bovine spongiform encephalopathy (BSE) is transmitted by the oral route. However, the impacts of anaerobic fermentation processes in cattle on the stability of BSE-associated prion protein (PrP^{Sc}) are still unresolved. In this study, experiments were designed to assess the ability of complex ruminal and colonic contents of bovines to degrade BSE-derived PrP^{Sc}. No significant decrease in PrP^{Sc} levels in BSE brain homogenates was detected by Western blotting after up to 66 h of co-incubation with intestinal fluids. These results indicate that BSE-associated PrP^{Sc} survive gastrointestinal digestion processes in cattle and might be excreted via faeces.

Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are fatal neurodegenerative disorders affecting animals and humans. According to the prion hypothesis, TSEs are caused by infectious agents known as prions, which consist mainly, if not entirely, of an abnormal disease-associated form of the prion protein (PrP) referred to as PrP^{Sc} (Prusiner, 1998). TSEs are characterized by the conversion of host-encoded cellular prion proteins (PrP^{C}) into its pathogenic isoforms (PrP^{Sc}), which accumulate in the central nervous system. The change in protein folding structure leads to the characteristic resistance of PrP^{Sc} towards inactivation. As a correlation between TSE infectivity and the presence of PrP^{Sc} has been shown, PrP^{Sc} is considered as a reliable marker of TSE infection (Beekes et al., 1996; Wadsworth et al., 2001). The biochemical detection of PrP^{Sc} remains the gold standard for the diagnosis of prion diseases.

Scrapie of sheep, chronic wasting disease of cervids, bovine spongiform encephalopathy (BSE) of cattle and variant Creutzfeldt–Jakob disease (vCJD) of humans are acquired prion diseases that are caused by the direct exposure to TSE agents. Evidence suggests that these diseases

are induced by ingestion of prions and subsequent invasion of the host via the alimentary tract (Bruce et al., 1997; Weissmann et al., 2002). An oral route of infection is commonly assumed to be important in the natural pathogenesis of BSE following the ingestion of infected tissues via contaminated feed (Wilesmith et al., 1988). However, the fate of the infectious PrP^{Sc} during polygastric digestion of ruminants still remains unclear. In a previous study, we reported on the ability of complex ruminal and colonic microbiota of cattle to degrade scrapie (263K)-associated PrP^{Sc} down to immunochemically undetectable levels under physiological anaerobic conditions, suggesting that the majority of PrP^{Sc} is readily digested (Scherbel et al., 2006). Recently, a study about the digestion of BSE-derived PrP^{Sc} in the sheep intestine was published. It was shown that degradation of PrP^{Sc} in the BSE inoculum had a significantly different pattern when compared with scrapie-derived PrP^{Sc}, with the BSE PrP^{Sc} degrading more rapidly (Dagleish et al., 2010). On the other hand, reports on the excretion of infectious prions in faeces of deer (Tamguney et al., 2009) and the detection of prions in the faeces of sheep naturally infected with scrapie (Terry et al., 2011) suggest that PrP^{Sc} survives gastrointestinal digestion processes in ruminants. Based on our initial findings, a corresponding follow-up study was performed to assess the ability of complex ruminal and colonic microbiota of cattle to degrade BSE-associated PrP^{Sc} in order to gain more information about the fate of PrP^{Sc} during polygastric digestion.

Materials and Methods

Therefore, a 20% BSE-infected brain homogenate (w/v) in physiological buffered saline was produced from a brain stem tissue pool from classical German BSE cases. All homogenates were stored in aliquots at −70°C until use. Rumen content and the ligatured section of the *colon ascendens* from healthy fattened beef bulls ($n = 4$) were taken under sterile conditions immediately after slaughtering. A 10% homogenate of the active microbiota of rumen and colon contents was prepared with sterile mineral salt buffer solution of McDougall in the absence and presence of soluble carbohydrates, for example maltose, xylose and soluble starch (Scherbel et al., 2006). Degradation assays were conducted using normal or BSE-infected brain homogenates and bovine gastrointestinal contents as described previously. Briefly, ruminal and colonic homogenates were filtrated to remove crude particles, and the samples were prepared in the ratio of 10 to 1 (vol/vol), concerning intestinal homogenate to BSE brain homogenate. Immediately after sample preparation (zero hour samples), aliquots were taken and stored at −70°C until further treatment. Incubation was carried out at 37°C for up to 66 h under anaerobic conditions. Samples were taken after 20, 40 and 66 h of incubation with rumen and colon contents. Buffer solution of McDougall with the addition of BSE brain homogenate represented the positive control. Samples were analysed at the selected time points in Western blots.

The immunochemical detection of PrP^{Sc} was accomplished after proteinase K digestion for 60 min at 37°C at a final concentration of 100 μg/ml. Proteins were subjected to electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Schaller et al., 1999). PrP^{Sc} was detected by immunostaining with monoclonal anti-prion antibody 6H4 (Prionics, Schlieren, Switzerland) in combination with a secondary antibody conjugated to horseradish peroxidase (Dianova, Hamburg, Germany). The immunochemical reactions were visualized using a highly sensitive chemiluminescence-based detection technique, the ECL detection system (Amersham Bioscience, Freiberg, Germany), and the signals were recorded on X-ray films. Densitometrical analyses were performed with ImageJ software (National Institutes of Health, Bethesda, MD, USA). Band intensities were calculated by plotting the total areas of the PrP^{Sc} bands on Western blots. Time zero sample signals were defined as 100% values. PrP^{Sc} signals before and after incubation were compared, and the changes were displayed in graphical form.

Results

In vitro degradation assays of infectious BSE-associated PrP^{Sc} with the complex microbiota of bovine rumen and colon were established under different conditions according to our previous study. However, the exposure of PrP^{Sc} to intestinal contents of cattle for up to 66 h did not result in its degradation in any instance. Densitometric analyses revealed no significant reduction in PrP^{Sc} signals in comparison with the control (Fig. 1). Based on our earlier experimental data, which indicated that an effective degradation of PrP^{Sc} is dependent on the presence of soluble carbohydrates, these supplements were added to selected samples to enhance microbial degradation capacity. However, no changes in PrP^{Sc} levels on Western blots were detected after an incubation period for 40 h with ruminal fluid (Fig. 2a, lanes 1–3) and colonic content (Fig. 2b, lanes 1–6), respectively.

Discussion

There are currently no clear-cut data available concerning the stability of cattle-derived BSE-associated PrP^{Sc} towards the fermentation processes in the bovine gastrointestinal tract. The mean retention time of digesta in the gastrointestinal tract in cattle is about 3 days (Schaefer

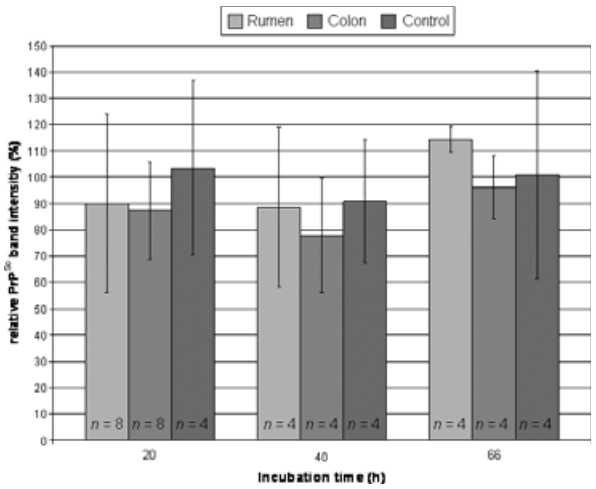

Fig. 1. Comparison of bovine spongiform encephalopathy (BSE)-derived prion protein (PrP^{Sc}) signal intensities in densitometric analyses. Band intensities were calculated by plotting the total areas of the PrP^{Sc} bands on Western blots. Time zero sample signals were defined as 100% values. PrP^{Sc} signals before and after incubation were compared, and the changes were displayed in graphical form. BSE brain homogenates were incubated under anaerobic conditions in the absence or presence of soluble carbohydrates with ruminal and colonic contents of cattle and buffer solution for 20, 40 and 66 h. Sample sizes are shown in the bars.

et al., 1978). Here, we demonstrate that incubation of BSE-associated PrP^{Sc} with both ruminal and colonic microbiota of cattle within an average feed passage rate through the bovine digestion tract did not result in a significant degradation of PrP^{Sc}, which confirms the notorious resistance of prions towards proteolytic digestion. These results are in contrast to the data obtained with laboratory hamster scrapie strain 263K–derived PrP^{Sc} exposed to the same conditions, although it was previously shown that BSE agents are less resistant to proteolytic digestion than scrapie (263K) strains (Kuczius and Groschup, 1999). Our findings implicate a great stability of BSE-associated PrP^{Sc} towards gastrointestinal digestion processes in cattle. Consequently, TSE strains from natural hosts might be more stable towards hydrolysis than laboratory prion strains. Peretz et al. (2006) also confirmed this theory by investigating different inactivation procedures of human and rodent prions.

However, several *in vitro* incubation assays of scrapie prions together with intestinal contents of sheep lead to the degradation of PrP^{Sc} in Western blot analyses (Dagleish et al., 2010; Jeffrey et al., 2006). Lately, it was shown that BSE-derived PrP^{Sc} is less protease resistant than scrapie-derived PrP^{Sc} by *in vitro* incubation with sheep gut contents (Dagleish et al., 2010). In contrast, our experiments

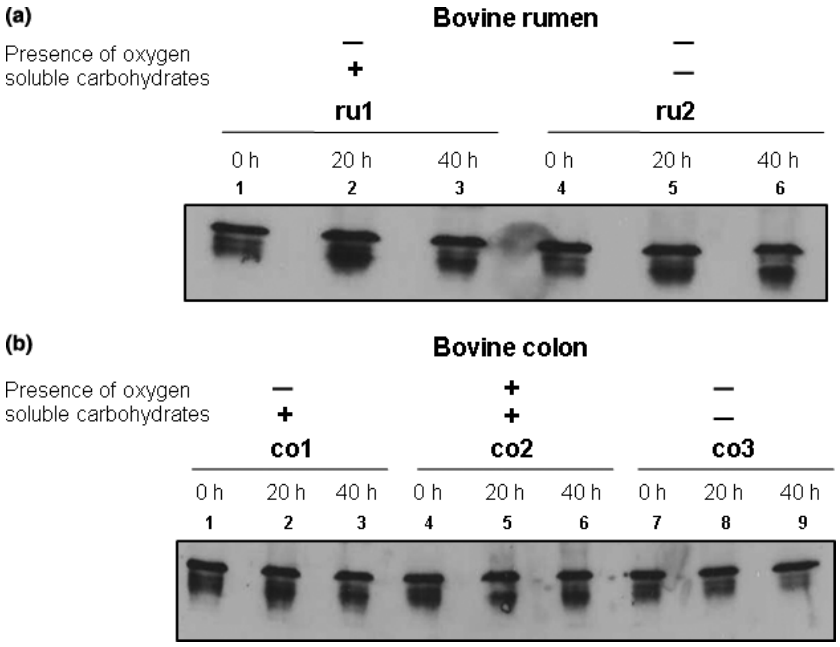

Fig. 2. *In vitro* bovine spongiform encephalopathy (BSE)-associated prion protein (PrP^{Sc}) degradation assay by gut microbiota of bovines. (a) Complex ruminal microbiota of cattle was incubated with BSE-infected brain homogenate in mineral salt buffer solution of McDougall under anaerobic conditions either in the presence (lanes 1, 2 and 3) or in the absence (lanes 4, 5 and 6) of soluble carbohydrates for 0, 20 and 40 h. (b) Complex colonic microbiota of cattle was incubated with BSE-infected brain homogenate in mineral salt buffer solution of McDougall under anaerobic (lanes 1, 2 and 3) and aerobic (lanes 4, 5 and 6) conditions in the presence of soluble carbohydrates for 0, 20 and 40 h. Complex colonic microbiota of cattle was incubated with BSE-infected brain homogenate in mineral salt buffer solution of McDougall under anaerobic conditions in the absence of soluble carbohydrates for 0, 20 and 40 h (lanes 7, 8 and 9).

revealed that BSE prions are notoriously resistant towards proteolytic digestion in the bovine alimentary tract. Simulating the authentic *in vivo* conditions, degradation studies were performed in an anoxic environment contrary to the previous studies. Above all, the results presented here are in accordance with the study of Nicholson et al. (2007) who evaluated the potential of rumen-simulating conditions to reduce PrP^{Sc} levels. Scrapie brain material was incubated up to 24 h under anaerobic conditions with rumen fluid freshly obtained from a fistulated cow. This treatment did not result in a reduction in PrP^{Sc} levels in Western blot analyses analogous to our experiments with BSE brain material. Nonetheless, differences in the diets of animals might explain the contradictious results of various incubation experiments, because feed and feed additives directly influence the composition of microbial gut communities.

The work presented here describes for the first time the combination of BSE-derived PrP^{Sc} with intestinal contents of the natural host. These results indicate that BSE-associated PrP^{Sc} survive gastrointestinal digestion processes in cattle and might be shed via faeces. Shedding of PrP^{Sc} in the excrements was already demonstrated in feeding experiments with scrapie-infected material to hamsters (Kruger et al., 2009), and the excretion of BSE and scrapie prions in stools from orally inoculated mice was also described (Maluquer de et al., 2008). Moreover, it was shown that faeces from infected sheep (Terry et al., 2011) and deer (Tamguney et al., 2009) are potential sources of contamination of the environment.

Our results now add further evidence that orally fed BSE prions can traverse the gastrointestinal tract of bovines without substantial PrP^{Sc} degradation.

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