Molecular analysis of Irish sheep scrapie cases

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Different strains of transmissible spongiform encephalopathies in humans and rodent models are associated with the accumulation of PrPSc of distinct molecular characteristics. These characteristics include glycosylation profiles, fragment sizes and long-term resistance of PrPSc to proteinase K. The first objective of this study was to determine the applicability of these criteria to characterize and differentiate sheep scrapie PrPSc and bovine spongiform encephalopathy (BSE) PrPSc. PrPSc in sheep scrapie samples from Ireland had clearly distinct molecular characteristics to PrPSc in cattle BSE samples using a monoclonal antibody (MAb P4) directed to position 89–104 of ovine PrP using either brain homogenates or semi-purified scrapie-associated fibrils. Similar glycoprofiles were found when analysing scrapie PrPSc in six different CNS regions (thoracic spinal cord, thalamus, basal ganglia, mediobasal hypothalamus, medulla oblongata and cortex). While the long-term resistance results using a different monoclonal antibody (raised to ruminant PrP positions 145–163; MAb L42) were similar to the results obtained with MAb P4, different glycotyping results were obtained. Given the variation in glycosylation patterns using different antibodies, we conclude that standardization of methodology and antibodies is crucial to the applicability of molecular analysis of ruminant BSE and scrapie samples.

Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative diseases characterized by the neuronal accumulation of an abnormal isoform of the prion protein (Oesch et al., 1985; Prusiner & DeArmond, 1994). While the normal (PrP°C) and abnormal (PrPSc) isoforms of the prion protein have the same amino acid sequence and molecular mass, they have dramatically different three-dimensional structure and biochemical characteristics. The three-dimensional structure of PrP°C is characterized by a high α-helix content (Riek et al., 1996), all or part of which undergoes a post-translational modification to β-sheet in PrPSc (Pan et al., 1993; Pergami et al., 1996; Safar et al., 1993). While biochemically, PrP°C (33–35 kDa) is completely hydrolysed with protease treatment, PrPSc is partially resistant to proteinase K as 62 N-terminal amino acids are cleaved leaving a core fragment of approximately 141 amino acids (27–30 kDa) unhydrolysed (Oesch et al., 1985).

Recent evidence suggests that bovine spongiform encephalopathy (BSE) has been transmitted from cattle to humans and caused variant Creutzfeldt–Jakob disease in the human population (Hill et al., 1997; Bruce et al., 1997). Further concerns have been raised about the possibility of natural transmission of the BSE agent to sheep, as experimental studies have identified that sheep develop clinical signs indistinguishable from conventional scrapie following oral infection with BSE (Foster et al., 1993). Therefore, in the European Union efforts to characterize sheep scrapie cases are being intensified in order to exclude underlying BSE infections.

A number of murine, hamster and mink scrapie strains have been established by serial passage of natural prion infectivity in animals (Kimberlin & Walker, 1978; Kimberlin et al., 1989; Bessen & Marsh, 1992, 1994; Dickinson & Meikle, 1971; Bruce et al., 1991). To date, the recognized method for distinguishing TSE strain types is the identification of differences in the incubation times and in pathological lesions in the brains of the infected mice (Fraser & Dickinson, 1973; Bruce & Dickinson, 1973).
Little is currently known about the natural strain diversity in sheep populations of different countries. Two research groups have reported on their findings in British scrapie cases by biochemically analysing the PrP\textsuperscript{Sc} glycoform ratios and fragment sizes (Hill et al., 1998; Hope et al., 1999). Both groups considered the degree of variation found to reflect the diversity of different strain types in the country. In contrast, little diversity and PrP\textsuperscript{Sc} glycoprofiles reminiscent of those of BSE prions were found in French scrapie cases (Baron et al., 1999).

It has been clearly demonstrated in mice that the host genotype can have a profound influence on the phenotypic characteristics of each TSE strain, in terms of incubation time period and brain lesion profile (Dickinson & Meikle, 1971; Bruce et al., 1991). Little is known about the influence of the host genetic factors on PrP\textsuperscript{Sc} characteristics in sheep. More than nine different alleles encoding PrP have been seen in sheep. Three polymorphisms at positions 136 [valine (V) or alanine (A)], 154 [arginine (R) or histidine (H)] and 171 [glutamine (Q), histidine (H) or arginine (R)] are linked to susceptibility or resistance to scrapie infection.

The first objective of this study was to identify the range of PrP\textsuperscript{Sc} glycoform patterns in scrapie sheep with different genotypes, ages and clinical signs from Ireland. The second objective was to determine the impact of the methodology used, such as tissue sample source, preparation protocols and antibodies used. Moreover, we compared the glycoform patterns with the genotyping results to identify if specific PrP\textsuperscript{Sc} types are found in sheep of specific genotypes and if the sheep genotypes influence the molecular characteristics of PrP\textsuperscript{Sc}.

Finally, analysis of 12 BSE brain samples from cattle which had come down with the disease in Great Britain, Ireland, Switzerland or Germany were included in order to reveal whether the scrapie cases harbour PrP\textsuperscript{Sc} of similar molecular characteristics to BSE.

\section*{Methods}

\textbf{Animals and tissue selection.} Sixteen Irish animals were selected from the Irish Database, which records breed, genotype, age at onset of clinical signs, brain lesion profile and clinical behaviour (McElroy et al., 2000). All animals were Suffolk crosses. Brain tissues from five animals with PrP\textsubscript{AA}139\textsubscript{RR}142\textsubscript{QQ}151; five animals with PrP AA\textsubscript{139}RR\textsubscript{142}QQ\textsubscript{151}; five animals with PrP VA\textsubscript{139}RR\textsubscript{142}QQ\textsubscript{151} and one animal with PrP VA\textsubscript{139}RR\textsubscript{142}OH\textsubscript{151} were selected with the greatest variation in clinical behaviour and age at onset of clinical signs.

To determine if there were differences in PrP\textsuperscript{Sc} molecular characteristics from different brain regions, tissue samples were analysed from the thoracic spinal cord, thalamus, basal ganglia, mediobasal hypothalamus, medulla oblongata and cortex. To determine if different glycoform patterns are present in sheep with different genotypes, age at onset of clinical signs, brain lesion profile and behaviour, the cerebellum was analysed.

\textbf{Tissue preparation.} For each isolate brain tissues were weighed and homogenized in PBS containing 0.5\% NP-40 and 0.5\% sodium deoxycholate as previously described (Collinge et al., 1996; Kuczius et al., 1998) and sonicated for 2 min. Residual cell debris was removed by centrifugation at 2000 r.p.m. for 5 min and the supernatants (10\% w/v homogenates) were stored at $-20^\circ$C.

Pathological prion proteins were purified using a modified protocol as previously described (Diringer et al., 1983). In summary, brain tissue was homogenized in brain lysis buffer (10 mM sodium phosphate containing 10\% N-laurylsarcosine, 3 mM PMSF and 3 mM N-ethylmaleimide) and sonicated for 1 min. Insoluble fragments were removed at 17000 r.p.m. for 30 min and scrapie-associated fibrils (SAFs) were pelleted at 48000 r.p.m. for 2 h in a further centrifugation step. Pellets were resolubilized in 16 mM Tris buffer (pH 7.4) and incubated at 37\°C for 30 min. After adding potassium iodide buffer (10 mM Tris buffer pH 7.4, 15\% potassium iodide, 60 mM sodium thiiosulfate) the pellet was incubated at 37\°C for a further 30 min. Potassium iodide buffer (10\%) was added and SAFs were pelleted at 55 000 r.p.m. for 1 h. Supernatants were removed and pellets were resolubilized in PBS and then stored at $-20^\circ$C.

\textbf{Treatment with proteinase K.} Homogenates or SAFs were treated with proteinase K (50 \textmu g/ml) and proteins hydrolysed for 1 h at 37\°C, unless otherwise indicated. The digestion was stopped by the addition of 5 mM PMSF (Boehringer).

\textbf{Immunoblot analysis.} Western blot analysis was performed by SDS–PAGE in 13\% or 16\% gels (mini-protein II, Dual Slab Cell, Biorad) as described before (Groschup & Pfaff, 1993). After addition of SDS-loading buffer, samples were boiled for 5 min before loading the gel. Proteins were electrobotted onto Immobilon P-membranes (Millipore) using a semi-drying blotting system (Biorad). Membranes were blocked in PBS containing 0.1\% Tween-20 and 5\% non-fat milk powder for 1 h. Detection of PrP occurred with PrP-specific monoclonal antibodies MAb P4 or MAb L42. These antibodies were raised to amino acid sequences of sheep PrP of 98–106 and 145–163, respectively (Harmeyer et al., 1998). Horseradish peroxidase-conjugated affinity-purified goat anti-mouse IgG (Dianova) served as detection antibody. The membranes were developed using a chemiluminescence enhancement kit (ECL, Amersham) and the proteins were visualized on high performance luminescence detection films (Hyperfilm ECL, Amersham) or recorded electronically by photo imager techniques.

To glycoform PrP isolates, the banding intensities of the non-, mono- and di-glycosylated isoforms were determined as previously described (Kuczius et al., 1998). Signal bands were scanned on a chemiluminescence photo imager (Fujiﬁlm) using the CSC program. Intensities of the three
resulting protein bands were determined using the TINA2.0 program (Raytest). The combined signals were defined as 100% and the contribution of each band was calculated in percent.

**Impact of metal ions on PrP<sub>Sc</sub> fragment sizes.** Homogenate samples were treated with proteinase K as described above. In order to determine whether the presence or absence of copper ions influences fragment sizes of the partially cleaved PrP<sub>Sc</sub>, reactions were performed in the presence or absence of copper sulfate (25 μM).

**Quantitative and statistical analysis.** For calculation of the banding intensities in long-term proteinase K-treated samples, total signals of the di-, mono- and non-glycosylated PrP<sub>Sc</sub> isoforms were defined as 100% for each isolate after hydrolyses for 1 h. To eliminate the possibility of artefacts due to differences in the sample/gel preparation during SDS–PAGE separation, a minimum of four separate runs were performed per homogenate or SAF preparation. Standard errors were calculated from means of the gel runs of the individual animals tested.

**Results**

**PrP<sub>Sc</sub> glycootyping of sheep scrapie and cattle BSE cases**

To compare the PrP<sub>Sc</sub> glycotyping patterns between scrapie-infected individuals with different genotypes, cerebellum homogenates were treated with proteinase K, separated by SDS–PAGE and immunoblotted using the monoclonal antibody MAb P4. Similar glycosylation patterns were observed in the PrP<sub>Sc</sub> from the 16 Irish scrapie-infected sheep (Figs 1 and 2a, P < 0.05). In all cases, the majority of PrP<sub>Sc</sub> was di-glycosylated (45–55%), followed by the mono-glycosylated (30–35%) and the non-glycosylated fraction (15–22%). Hence, similar glycosylation patterns were detected in sheep regardless of genotype and clinical signs. Of significant interest was the observation that the glycotype pattern for the PrP<sub>Sc</sub> from BSE-infected cattle (di-glycosylated 60–70%; mono-glycosylated 20–30%; non-glycosylated 5–12%) using MAb P4 (Fig. 2b) was clearly distinct from PrP<sub>Sc</sub> from the scrapie-infected sheep.

**Impact of sample preparation on glycootyping results**

To determine if the method of purification could have an influence on the glycosylation profile, cerebellum samples from all scrapie-infected animals were prepared as SAFs and as crude homogenates. As shown in Fig. 2(a, b) and determined by statistical analysis, PrP<sub>Sc</sub> obtained from both preparations contained the same glycotyping distribution.

**Glycosylation profile in tissues from different regions of the brain**

The impact of the anatomical source of brain tissue used on the PrP<sub>Sc</sub> glycosylation profile was determined by analysing different brain regions (thalamus, thoracic spinal cord, basal ganglion, mid-brain, medulla and cortex) from seven sheep. The results are presented in Fig. 3. As revealed by statistical means, glycootyping ratios between di-glycosylated, mono-glycosylated and non-glycosylated forms were similar be-

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**Figure 1.** Example of an immunoblot depicting PrP<sub>Sc</sub> bands purified as SAFs from cerebellum tissue samples of Irish scrapie sheep. Glycoforms were stained using MAb P4 and antibody binding was visualized by chemiluminescence blackening of conventional film.

**Figure 2.** Proportions of PrP<sub>Sc</sub> glycoforms in 16 different sheep following either tissue preparation by homogenates (a, c) or SAFs (b). Immunoblots were developed using MAb P4 (a, b) or MAb L42 (c). The percentages of di- ( ), mono- ( ■) and non-glycosylated ( ▲) PrP<sub>Sc</sub> isoforms are presented as means ± SDs for individual animals. Animal identification numbers are given on the x-axis and correspond to the following genotypes: AARRQH (39, 40, 42, 49 and 50), AARRQQ (38, 48, 53, 46, 47), VARRQH (29) and VARRQQ (23, 25, 33, 44, 54). The BSE glycoprofile pattern represents the combined results of 12 BSE-diseased animals that were analysed. The value for each animal was evaluated from a minimum of five separate gels.
cerebellum and/or brain stem). In comparison to the glycoype profile obtained from sheep PrP\textsuperscript{Sc}, a unique and highly reproducible BSE PrP\textsuperscript{Sc} glycotyping pattern was observed which was the same in all anatomical regions of the brain examined (data not shown).

**PrP genotypes**

PrP\textsuperscript{Sc} glycotytypes obtained for scrapie sheep carrying different PrP genotypes were generally similar (Fig. 4).

**PrP\textsuperscript{Sc} molecular masses**

No or only minor differences were detected in the molecular masses for partially proteinase K-digested PrP\textsuperscript{Sc} of Irish scrapie-infected sheep using MAb P4, which were hardly distinguishable from one another, given the inter- and intra-gel variations in the gel sieve separation observed frequently. In order to exclude artefacts, PrP\textsuperscript{Sc} cleavage experiments were performed in the absence of copper-binding substances, such as EDTA. Moreover, proteolytic cleavage experiments were performed in the presence or absence of copper ions – yet without detectable effect on the molecular masses of sheep scrapie PrP\textsuperscript{Sc}.

**Impact of detection antibody used**

To determine the impact of using a different antibody on the glycotyping profile, molecular mass and proteinase K
Irish sheep scrapie cases

Fig. 4. Proportions of PrPSc glycoforms in the cerebellum of animals of different genotypes as obtained by quantitative photo imager techniques. The percentages of di- (◆), mono- (■) and non-glycosylated (▲) PrPSc isoforms are presented as means ± SDs for individual genotypes. These values include five animals from each of the genotypes AARRQH, AARRQQ and VARRQQ, and one animal of the genotype VARRQH. MAb P4 was used as detection antibody. The value for each animal was evaluated from a minimum of five separate gels.

resistance (see below), another monoclonal antibody, MAb L42, which binds to a different epitope of ovine PrPSc (145–163) was used and the results compared to those obtained with MAb P4. The glycotyping patterns obtained using MAb L42 were different from the patterns observed with MAb P4 (Fig. 2c). The ratio of the di-glycosylated band of PrPSc was generally higher (up to 65%) and the results were not as uniform as those obtained using MAb P4. Moreover, a number of scrapie cases exhibited PrPSc glycoforms which were indistinguishable from those of BSE PrPSc.

Long-term proteinase K resistance

Patterns of long-term resistance to proteinase K digestion were identified in all scrapie cases, and were independent of the antibody used, MAb P4 or MAb L42. They were distinct from the patterns observed for the BSE-derived samples. Scrapie PrPSc was significantly more robust and generally more than 50% of the initial signal was retained after 6 h of proteinase K exposure at 50 µg/ml. In contrast, less than 50% of the BSE PrPSc signal was retained under the same experimental conditions (Fig. 5).

Discussion

In this study, PrPSc from 16 sheep from Ireland naturally infected with scrapie as well as of 12 cows infected with BSE from the UK, Switzerland, Ireland and Germany, were characterized in respect to their glyctype, molecular mass, and long-term proteinase K resistance. Using a monoclonal antibody designated MAb P4, which was raised to amino acids 89–104 of ruminant PrP, similar glycosylation and proteinase K resistance profiles were observed in all scrapie cases. These profiles were different from those obtained for cattle-derived BSE PrPSc. However, an interesting finding was that the choice of antibody used for PrPSc detection influenced the glyco-otyping profiles obtained. Using MAb L42 (binding to an epitope in the vicinity of amino acid 148 of ruminant PrP) instead of MAb P4 yielded slightly different BSE and scrapie PrPSc glycoforms, which were indistinguishable.

As shown before, murine TSE strains display non-uniform long-term proteinase K resistances (Kuczius & Groschup, 1999). We have therefore exposed PrPSc of 16 scrapie sheep as well as PrPSc produced in BSE-diseased cattle for prolonged
glycotypes were comparatively small and little diversity in the situation, little variation in the glycotypes and fragment sizes before the years 2000–2001. Data from strain typing experiments will not be available results obtained. However, for the samples used in this study antibody used can have a remarkable effect on the glycotyping lesion profile scoring assays in mice) become available. This is with caution until biotyping assay data (incubation time and technology and the lack of understanding of the underlying supply and accessibility. However, given the elaborate methodology is by far superior in regard to a linear range of measurements than densitometer scanning of blackened films.

Recent analysis of PrP\textsuperscript{Sc} (glycoform ratios and fragment sizes) following treatment with proteinase K in British scrapie cases demonstrated a remarkable diversity of profiles, i.e. various glycosylation patterns combined with a variety of molecular masses of the proteinase K-cleaved unglycosylated PrP\textsuperscript{Sc} fragments. It was argued that these molecular patterns could reflect the diversity of field sheep scrapie strains in the UK (Hill \textit{et al.}, 1998; Hope \textit{et al.}, 1999). In contrast to the UK situation, little variation in the glycotypes and fragment sizes were identified in all of the Irish scrapie cases in this study. Even when using Mab L42 the observed variations in the glycotypes were comparatively small and little diversity in the molecular masses was noticeable. Almost uniform glycoprofiles have recently been described for French scrapie cases (Baron \textit{et al.}, 1999), which are generally in line with the results found in our study.

Probably the most important question is whether BSE is present in sheep. Tissue samples from BSE-infected sheep were not analysed in the current data sets, because of their limited supply and accessibility. However, given the elaborate methodology and the lack of understanding of the underlying mechanisms, biochemical typing results should be interpreted with caution until biotyping assay data (incubation time and lesion profile scoring assays in mice) become available. This is particularly highlighted by the observation that the choice of antibody used can have a remarkable effect on the glycotyping results obtained. However, for the samples used in this study data from strain typing experiments will not be available before the years 2000–2001.

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