Distinct glycoform ratios of protease resistant prion protein associated with PRNP point mutations

Andrew F. Hill, Susan Joiner, Jonathan A. Beck, Tracy A. Campbell, Andrew Dickinson, Mark Poulter, Jonathan D. F. Wadsworth and John Collinge

MRC Prion Unit and Department of Neurodegenerative Disease, Institute of Neurology, University College London, National Hospital for Neurology and Neurosurgery, Queen Square, London, UK

1Present addresses: Department of Biochemistry & Molecular Biology, Bio21 Molecular Science and Biotechnology Institute and Department of Pathology, University of Melbourne, Parkville, Victoria 3010, Australia

Correspondence to: Professor John Collinge, MRC Prion Unit and Department of Neurodegenerative Disease, Institute of Neurology, University College London, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG, UK
E-mail: j.collinge@prion.ucl.ac.uk

Inherited prion diseases are neurodegenerative disorders caused by autosomal dominant mutations in the human prion protein gene (PRNP). Kindred with inherited prion disease can show remarkable phenotypic variability that has yet to be explained. Here we report analysis of protease resistant disease-related prion protein (PrPSc) isoforms from a range of inherited prion disease cases (point mutations P102L, D178N, E200K and 2-, 4- and 6-octapeptide repeat insertions) and show that the glycoform ratios of PrPSc associated with PRNP point mutations are distinct from those observed in sporadic, iatrogenic and variant Creutzfeldt–Jakob disease. Patients with the same PRNP mutation can also propagate PrPSc with distinct conformations. These data extend the spectrum of recognized PrPSc types seen in human prion diseases and provide further insight into the generation of diverse clinicopathological phenotypes associated with inherited prion disease.

Keywords: Creutzfeldt-Jakob disease; prion disease; prion protein; fatal familial insomnia

Abbreviations: CJD = Creutzfeldt–Jakob disease; FFI = fatal familial insomnia; GSS = Gerstmann–Straussler–Scheinker syndrome; MHs = methionine homozygotes; vCJD = variant CJD


Introduction

Human prion diseases are fatal, transmissible neurodegenerative disorders that include Creutzfeldt–Jakob disease (CJD), variant CJD (vCJD), Gerstmann–Straussler–Scheinker syndrome (GSS), familial fatal insomnia (FFI) and kuru (Collinge, 2001). The central feature of prion diseases is the post-translational conversion of cellular prion protein (PrPc) to an abnormal protease-resistant isoform designated PrPSc (Prusiner, 1998; Collinge, 2001). Inherited prion diseases account for ~15% of human cases and are caused by autosomal dominant mutations in the human prion protein gene (PRNP) (Collinge, 2001; Wadsworth et al., 2003). Traditionally, inherited prion diseases have been classified by the presenting clinical or pathological syndrome, falling into three main sub-divisions of either GSS, CJD or FFI (Collinge and Palmer, 1997; Collinge, 1998; Kovacs et al., 2002; Gambetti et al., 2003; Harder et al., 2004). Remarkably, however, some families show extensive phenotypic variability, which can encompass both CJD- and GSS-like disease as well as other cases that do not conform to either CJD or GSS phenotypes (Collinge et al., 1992; Hainfellner et al., 1995; Mallucci et al., 1999; Kovacs et al., 2002).

Within the framework of the protein-only hypothesis of prion propagation, distinct clinical and neuropathological phenotypes are thought to be determined by the propagation of distinct disease-related PrPSc isoforms with divergent physicochemical properties (Bessen and Marsh, 1994; Collinge et al., 1996; Parchi et al., 1996; Gambetti et al., 2003; Hill et al., 2003; Zanusso et al., 2004). To date we have identified four
major types of human PrPSc associated with sporadic and acquired human prion diseases that can be differentiated on immunoblots after limited protease K digestion of brain homogenates (Collinge et al., 1996; Wadsworth et al., 1999; Hill et al., 2003). PrPSc types 1–3 are seen in classical (sporadic or iatrogenic) CJD brain, whereas type 4 PrPSc is uniquely seen in vCJD brain (Collinge et al., 1996; Wadsworth et al., 1999; Hill et al., 2003). An earlier classification of PrPSc types seen in classical CJD described only two banding patterns (Parchi et al., 1996) with PrPSc types 1 and 2 that we describe corresponding with the type 1 pattern of Gambetti and co-workers (2003), and our type 3 fragment size corresponding to their type 2 pattern (Parchi et al., 1997, 1999). Whereas type 4 PrPSc is readily distinguished from the PrPSc types seen in classical CJD by a predominance of the di-glycosylated PrP glycoform, type 4 PrPSc also has a distinct proteolytic fragment size (Hill et al., 2003), although this is not recognized by the alternative classification, which designates type 4 PrPSc as type 2b (Parchi et al., 1997).

Efforts to produce a unified international classification and nomenclature of human PrPSc types has been complicated by the fact that the N-terminal conformation of some PrPSc subtypes seen in sporadic CJD can be altered in vitro via changes in metal-ion occupancy (Wadsworth et al., 1999; Hill et al., 2003) or solvent pH (Zanusso et al., 2001; Notari et al., 2004). Although it has recently been proposed that pH alone determines PrPSc N-terminal structure (Notari et al., 2004), this interpretation has not been supported by other studies (Lewis et al., 2005; Polymenidou et al., 2005), and strain-specific PrPSc conformations show critical dependence upon the presence of copper or zinc ions under conditions where pH 7.4 is strictly controlled (Wadsworth et al., 1999). Although agreement has yet to be reached on methodological differences, nomenclature and the biological importance of relatively subtle biochemical differences in PrPSc, there is strong agreement between laboratories that phenotypic diversity in human prion disease relates to the propagation of disease-related PrP isoforms with distinct physicochemical properties (Collinge et al., 1996; Parchi et al., 1996, 1999, 2000; Wadsworth et al., 1999; Gambetti et al., 2003; Hill et al., 2003; Zanusso et al., 2004).

Polymorphism at residue 129 of human PrP [encoding either methionine (M) or valine (V)] powerfully affects genetic susceptibility to human prion diseases (Collinge et al., 1991; Palmer et al., 1991; Lee et al., 2001; Mead et al., 2003). Within a conformational selection model of prion transmission barriers (Collinge, 1999, 2001; Hill and Collinge, 2003) it is predicted that coding changes in PrP act to specify thermodynamic preferences for disease-related PrP conformations. Although a wealth of data from acquired or sporadic CJD indicates that residue 129 polymorphism critically dictates thermodynamic preferences for disease-related PrP isoforms (Collinge, 2001; Hill and Collinge, 2003; Hill et al., 2003; Hosszu et al., 2004; Wadsworth et al., 2004), the full spectrum of effects that different pathogenic PRNP mutations may have remains unclear. However, numerous studies of abnormal PrP isoforms in inherited prion diseases have established that the detection of PrPSc in the molecular mass range of ~21–30 kDa is by no means a consistent feature. Instead, some cases, in particular those in which amyloid plaques are a prominent feature, show smaller protease resistant fragments of ~7–15 kDa (Parchi et al., 1998; Piccardo et al., 1998, 2001; Tagliavini et al., 2001; Kovacs et al., 2002). PrPSc glycoforms in patients with FFI or GSS also show dramatic differences from PrPSc seen in sporadic CJD (Furukawa et al., 1998; Parchi et al., 1998; Cardone et al., 1999) and have been reported to be more similar to that of type 4 PrPSc seen in vCJD (Furukawa et al., 1998). In order to further define the spectrum of abnormal PrP isoforms seen in human prion disease we performed detailed analysis of protease-resistant PrP species in patients with different PRNP mutations, including those classified as CJD, GSS and FFI. Our findings indicate that pathogenic PrP point mutations may have a profound effect in specifying the physicochemical properties of disease-related PrP isoforms and provide a critical insight into the potential mechanisms underlying phenotypic variability in inherited prion disease.

**Methods**

**Biosafety**

All procedures were carried out in a microbiological containment level 3 facility with strict adherence to safety protocols.

**Selection of patients**

This study was performed under approval from the Institute of Neurology/National Hospital for Neurology and Neurosurgery Local Research Ethics Committee. Tissues were derived from an unselected series of patients with a neuropathologically proven diagnosis of inherited prion disease who had provided consent to use autopsy material for research purposes. Ethical permission for research on autopsy materials stored in the National CJD Surveillance Unit was obtained from Lothian Region Ethics Committee.

**Genetic analysis of PRNP**

DNA was extracted from frozen brain tissue or peripheral blood. PCR of the PRNP open reading frame was performed using previously described oligonucleotide primers, which were selected so that they did not overlay a polymorphism in the intron 5′ to the open reading frame, which prevents the amplification of some alleles (Palmer et al., 1996). The PCR fragments were size fractionated on 1.5% agarose gels to investigate insertions or deletions in the octapeptide repeat region. The PCR products were then sequenced using both strands on an Applied Biosystems 377 DNA sequencer. For haplotype analysis, oligonucleotide primers were designed to perform allele specific PCR (asPCR) between the M129V (ATG–GTG) transition and a second primer targeted either 5′ (A117V, P102L and OPRI haplotype analysis) or 3′ (D178N and E200K haplotype analysis). Haplotyping of N-terminal variations used reverse asPCR primers 5′-CTCATGGCACTTCCCCACGGCAT-3′ (methionine allele) and 5′-CTCATGGCACTTCCCCACGCACG-3′.
(valine allele), with forward primer 5'-ACCTGGGCTCTCGGACGAAG-3'. Annealing temperatures for met and val alleles were 67 and 69°C, respectively. Haplotyping of C-terminal variations used forward asPCR primers 5'-GGGCCCTGGGCGCTACA-3' (methionine allele) and 5'-GGGCCCTGGCCGCTACGG-3' (valine allele), with the reverse primer 5'-AAGAGAAAGAAAGAGTGAACAC-3'. Annealing temperatures in this case were 66°C for both alleles. Thermal cycling was performed using 95°C denature for 5 min, followed by 35 cycles of 95°C for 30 s denature, anneal for 30 s at the temperature described above and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. Allele specific PCR products were sequenced using asPCR primers on an ABI 377 sequencer.

**Immunoblot analysis**

10% (w/v) brain homogenates were prepared in lysis buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris–HCl, 0.5% sodium deoxycholate, 0.5% NP-40, pH 7.4) by passage through needles of decreasing diameter. The homogenates were cleared by centrifugation at 800 r.p.m. (100 g) in a microcentrifuge for 1 min. Samples of supernatant were treated with proteinase K (50 μg/ml final concentration unless stated otherwise) for 60 min at 37°C. The reaction was terminated by the addition of Pefabloc (Boehringer Mannheim) to 1 mM final concentration. An equal volume of SDS loading buffer (125 mM Tris–HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, 4% β-mercaptoethanol) was added and the samples boiled for 5 min before electrophoresis on 16% Tris–glycine gels (Invitrogen). The gels were electroblotted onto PVDF membrane (Immobilon-P, Millipore) and blocked for 1 h in phosphate-buffered saline (PBS)/0.05% Tween-20 (PBST) containing 5% non-fat milk powder. Membranes were incubated with anti-PrP monoclonal antibody 3F4 (Kascak et al., 1987) (Signet Pathology Inc) at 0.2 μg/ml final concentration in PBST for 1 h or overnight. After washing in PBST (45 min), membranes were incubated with an alkaline phosphatase conjugated goat anti-mouse IgG antibody (Sigma) diluted 1 : 10 000 in PBST. Following washing in PBST (45 min), membranes were developed in chemiluminescent substrate (Atto phospho, Promega) and visualized on a Storm 840 phosphoimager (Molecular Dynamics). The glycoform profiles were quantitated using Imagequant software (Molecular Dynamics) to measure the relative intensity of each PrPSc band, which is then expressed as a percentage of the total signal. Each case of inherited prion disease suitable for analysis was analysed at least twice. Statistical analysis was performed using the INSTAT program (GraphPad Software, San Diego). Immunoblotting with anti-PrP monoclonal antibody 3F4 and enhanced chemiluminescence was performed as described previously (Wadsworth et al., 2001).

**High sensitivity immunoblotting**

Sodium phosphotungstic acid precipitation of PrPSc from 10% tissue homogenate, proteinase K digestion and immunoblotting with anti-PrP monoclonal antibody 3F4 using high sensitivity chemiluminescence was performed as described previously (Wadsworth et al., 2001). This method facilitates highly efficient recovery and detection of PrPSc from tissue homogenate when present at levels 103–104-fold lower than those found in vCJD brain (Wadsworth et al., 2001; Joiner et al., 2002; Frosh et al., 2004).

**Results**

**PrP analysis of inherited prion disease cases in the absence of proteinase K digestion**

Immunoblotting of brain homogenate in the absence of proteinase K digestion showed no discernible difference in the size of full length PrP from cases with PRNP point mutations (P102L, E200K and D178N) when compared with wild-type PrP in sporadic or vCJD (Fig. 1A). As expected however, similar analysis of inherited prion disease cases caused by 2-, 4- or 6-octapeptide repeat insertional mutations (OPRI) showed PrP species with a greater molecular mass than wild-type PrP, this being most apparent with the larger insert cases (Fig. 1B). Although theoretically all of the samples analysed should show closely similar levels of PrP, it should be emphasized that we are analysing post-mortem samples having intrinsic differences in post-mortem delay that can lead to degradation of PrPSc. The presence of truncated PrP fragments (particularly of ~20 kDa) generated by endogenous proteolytic activity in brain homogenate is well recognized (Jiménez-Huete et al., 1998) and levels of these can vary quite markedly between samples. Although an 8 kDa PrP species can sometimes be detected in non-proteinase K digested P102L brain homogenate (Piccardo et al., 1998; Zou et al., 2003) this was not a prominent characteristic of our series of patient samples.

**Fig. 1** Immunoblots of PrP in brain homogenate from classical CJD, vCJD and cases of inherited prion disease analysed without proteinase K digestion. A and B, respectively, show cases of inherited prion diseases associated with PRNP point mutations or octapeptide repeat insertions (OPRI). Immunoblots were developed with anti-PrP monoclonal antibody 3F4 using a chemiluminescent substrate.
PrP<sup>sc</sup> analysis in inherited prion disease cases

We analysed protease-resistant PrP species present in a series of patients with inherited prion disease. A total of 25 cases were studied of which 17 had PRNP point mutations and 8 had insertions in the octapeptide repeat region (OPRI mutations) (Table 1). Previous findings in sporadic CJD of detection of two apparently different PrP<sup>sc</sup> types in different regions of the same brain (Puoti et al., 1999; Head et al., 2004; Polymenidou et al., 2005) indicate the need to compare the same brain region from different patients for comparative PrP<sup>sc</sup> typing.

The samples used in our study were from frontal cortex and each sample was routinely analysed at least twice using appropriate controls of known PrP<sup>sc</sup> type on every immunoblot. Figure 2 illustrates the comparison in PrP<sup>sc</sup> fragment size and degree of glycosylation between classical CJD (types 1–3), vCJD (type 4) and inherited cases caused by octapeptide repeat insertion mutations (OPRI) or point mutations in the PRNP gene.

### PrP<sup>sc</sup> analysis of cases with octapeptide repeat insertion (OPRI) mutations

We examined frontal cortex from patients with three different OPRI mutations (2-OPRI, 4-OPRI and 6-OPRI). All were PRNP point mutations are designated by the wild-type amino acid preceding the codon number followed by the mutant residue using single letter amino acid nomenclature. (OPRI, octapeptide repeat insertion mutations).

<table>
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<th>PRNP haplotype (mutation – codon 129 genotype)</th>
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### PrP<sup>sc</sup> glycosylation in inherited prion diseases

We analysed protease-resistant PrP species present in a series of patients with inherited prion disease. A total of 25 cases were studied of which 17 had PRNP point mutations and 8 had insertions in the octapeptide repeat region (OPRI mutations) (Table 1). Previous findings in sporadic CJD of detection of two apparently different PrP<sup>sc</sup> types in different regions of the same brain (Puoti et al., 1999; Head et al., 2004; Polymenidou et al., 2005) indicate the need to compare the same brain region from different patients for comparative PrP<sup>sc</sup> typing.

The samples used in our study were from frontal cortex and each sample was routinely analysed at least twice using appropriate controls of known PrP<sup>sc</sup> type on every immunoblot. Figure 2 illustrates the comparison in PrP<sup>sc</sup> fragment size and degree of glycosylation between classical CJD (types 1–3), vCJD (type 4) and inherited cases caused by octapeptide repeat insertion mutations (OPRI) or point mutations in the PRNP gene.
All four cases that propagated PrPSc proteolytic fragments of K-resistant PrP species seen in P102L inherited prion disease typify both the spectrum and spatial variability of proteinase et al [1998] researchers (Parchi (Fig. 4). These data are consistent with those of other the 8 kDa PrP fragment was detected in the other three cases tease resistant PrP fragment (data not shown), whereas only kDa (Fig. 3B) together with variable levels of an 8 kDa pro-

PrPSc analysis in cases with PRNP point mutations

P102L

We examined seven P102L cases of which two were PRNP codon 129 heterozygotes and the other five were codon 129 MHs (Table 1). Analysis of multiple homogenates prepared from each specimen identified in four cases varying concentrations of proteinase K resistant PrP fragments of ~21–30 kDa (Fig. 3B) together with variable levels of an 8 kDa protease resistant PrP fragment (data not shown), whereas only the 8 kDa PrP fragment was detected in the other three cases (Fig. 4). These data are consistent with those of other researchers (Parchi et al., 1998; Piccardo et al., 1998) and typify both the spectrum and spatial variability of proteinase K-resistant PrP species seen in P102L inherited prion disease. All four cases that propagated PrPSc proteolytic fragments of ~21–30 kDa showed fragment sizes corresponding to either PrPSc type 1 or type 2 seen in sporadic CJD (Table 1 and Fig. 3B). In agreement with previous studies (Cardone et al., 1999; Furukawa et al., 1998; Parchi et al., 1998) analysis of the glycoform ratio of the PrPSc fragments of ~21–30 kDa from these patients revealed an abundance of diglycosylated PrP (Fig. 2C). However, densitometry showed that this glycoform ratio (high molecular mass glycoform, 40.46 ± 2.60%; low molecular mass glycoform, 39.18 ± 2.06%; unglycosylated, 20.36 ± 0.81%) differs significantly from PrPSc types 1–3 seen in classical CJD and from type 4 PrPSc seen in vCJD (Fig. 5).

A117V

A single PRNP codon 129 heterozygote patient with the A117V mutation was examined (Table 1). Following proteinase K digestion, PrPSc fragments of ~21–30 kDa were not detected but instead an 8 kDa protease-resistant PrP species was observed (Fig. 4). This finding correlates well with the findings of other researchers that only detected similar low molecular mass protease-resistant PrP species in A117V cases (Piccardo et al., 1998, 2001; Tagliavini et al., 2001).

D178N

We examined three patients with the D178N mutation all of which were of PRNP 129MM genotype (Table 1). Two of these cases propagated PrPSc with fragment sizes corresponding to type 2 PrPSc seen in sporadic CJD (Fig. 2D). The glycoform ratio of these PrPSc fragments showed an abundance of the di-glycosylated PrP glycoform (high molecular mass glycoform, 53.72 ± 4.88%; low molecular mass glycoform, 44.2 ± 2.07%; unglycosylated, 12.05 ± 1.36%), similar to those observed in the P102L cases, that was significantly different from PrPSc of the same type and codon 129 genotype seen in sporadic CJD (Fig. 5).
PrP<sup>Sc</sup> glycosylation in inherited prion diseases

Fig. 5 Ratio of three principal protease-resistant PrP glycoforms of ∼21–30 kDa seen in classical CJD, vCJD and cases of inherited prion disease. Data points represent the mean relative proportions of di-, mono- and unglycosylated PrP as percentage ± SEM. In some cases the error bars were smaller than the symbols used. The number of cases analysed were: sCJD type 1 PrP<sup>Sc</sup> 129MM (n = 17), sCJD type 2 PrP<sup>Sc</sup> 129MM, MM, MV, VV (n = 57), sCJD type 3 PrP<sup>Sc</sup> 129MM (n = 1), sCJD type 3 PrP<sup>Sc</sup> 129MV (n = 8), vCJD type 4 129MM (n = 30), P102L 129MM (n = 4), D178N 129MM (n = 2), E200K 129, MM, MV, VV (n = 5), OPRI mutations 129 MM, MV (n = 5). For PRNP point mutations P102L, E200K and D178N, there is a statistically significant difference in the proportions of di- and un-glycosylated PrP glycoforms when compared with PrP<sup>Sc</sup> types 1–3 in classical CJD (P < 0.0001) for the di- and un-glycosylated bands; unpaired t-test) and in the proportions of mono- and un-glycosylated PrP glycoforms compared with type 4 PrP<sup>Sc</sup> seen in vCJD (P < 0.004 for either glycoform; unpaired t-test). The PrP glycoform ratio in OPRI cases shows no significant difference from PrP<sup>Sc</sup> seen in sporadic CJD cases of the same codon 129 genotype (P > 0.1).

E200K

Six cases of inherited prion disease with the E200K mutation were examined. Three cases were PRNP codon 129 MHs, two were methionine/valine heterozygotes and one was a valine homozygote (Table 1). All three codon 129 MHs propagated PrP<sup>Sc</sup> with a fragment size corresponding to type 1 PrP<sup>Sc</sup> seen in sporadic CJD (Fig. 2E), accompanied by weak variable labelling of a ∼12 kDa protease resistant fragment (Fig. 4). One PRNP 129MV heterozygote propagated PrP<sup>Sc</sup> with a fragment size corresponding to type 2 PrP<sup>Sc</sup> seen in sporadic CJD (Fig. 3C) and the single PRNP 129VV homozygote propagated PrP<sup>Sc</sup> with a fragment size corresponding to type 3 PrP<sup>Sc</sup> seen in sporadic CJD (Fig. 3C and D). The other PRNP 129MV heterozygote patient showed PrP<sup>Sc</sup> with fragment sizes corresponding to both type 2 and type 3 PrP<sup>Sc</sup> seen in sporadic CJD (Fig. 3D). The presence of PrP<sup>Sc</sup> with two clearly defined proteolytic fragment sizes is also occasionally observed in brain homogenates from sporadic CJD cases (Parchi et al., 1999; Hill et al., 2003) and may represent the propagation of two different PrP<sup>Sc</sup> types in the same region of the brain (Puoti et al., 1999; Head et al., 2004; Polymenidou et al., 2005). The glycoform ratio of PrP<sup>Sc</sup> fragments seen in these E200K cases was similar to the P102L and D178N cases in this study (high molecular mass glycoform, 42.76 ± 1.59%; low molecular mass glycoform, 41.53 ± 2.17%; unglycosylated, 15.71 ± 1.52%), and again was significantly different to PrP<sup>Sc</sup> types 1–3 seen in sporadic CJD and type 4 PrP<sup>Sc</sup> seen in vCJD (Fig. 5). Thus in the six cases studied with the E200K mutation, we were able to observe all of the common PrP<sup>Sc</sup> fragment sizes previously reported in sporadic and iatrogenic prion disease (Collinge et al., 1996; Wadsworth et al., 1999; Hill et al., 2003) (Fig. 3C), but with the additional superimposition of a distinct glycoform ratio (Fig. 5). We also investigated the behaviour of E200K samples following proteinase K digestion in the presence of 25 mM EDTA (Wadsworth et al., 1999; Hill et al., 2003). All three PRNP 129MM homozygous cases with type 1 PrP<sup>Sc</sup> fragment size showed altered proteinase K digestion products after inclusion of EDTA during proteolysis, generating a fragment size corresponding to type 2’ generated from EDTA-treated type 1 PrP<sup>Sc</sup> in sporadic CJD (Fig. 6). In comparison, PrP<sup>Sc</sup> with type 2 fragment size from the codon 129 heterozygote case and PrP<sup>Sc</sup> with type 3 fragment size from the codon 129 valine homozygous case showed no alteration in the electrophoretic mobility of proteolytic fragments after digestion in the presence of EDTA (Fig. 6).

Analysis of lymphoreticular tissues for the presence of PrP<sup>Sc</sup>

Samples of lymphoreticular tissue were available for analysis from six patients, four with PRNP point mutations and two with a 6-OPRI insert mutation (Table 2). Using high sensitivity immunoblotting (Wadsworth et al., 2001) we were not able to detect PrP<sup>Sc</sup> in any of these tissues (Table 2). The...
Table 2 High sensitivity immunoblot analysis of lymphoreticular tissues from patients with inherited prion disease

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<td>E200K</td>
<td>6197</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>6 repeat OPRI</td>
<td>6354</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

The sensitivity of this method would detect PrPSc when present at levels >0.1% of the maximal level of PrPSc observed in necropsy vCJD tonsil (Wadsworth et al., 2001). *Tonsil biopsy specimen; no brain tissue available for analysis.

detection sensitivity of this method indicates that if PrPSc is present in lymphoreticular tissues from these cases of inherited prion disease then this is at a concentration <0.1% of the maximal level seen in necropsy vCJD tonsil (Wadsworth et al., 2001). To date, tonsil biopsy has shown 100% sensitivity and specificity for diagnosis of vCJD (Hill et al., 1999; Wadsworth et al., 2001; Frosh et al., 2004) and may allow diagnosis at an early clinical stage (Wadsworth et al., 2003). Our present findings suggest that the peripheral pathogenesis in inherited prion disease more closely resembles that of classical CJD, rather than vCJD, and therefore cases of inherited prion disease are unlikely to be detected by analysis of tonsil biopsy specimens.

Discussion

In this study we have shown that cases of inherited prion disease caused by the PRNP point mutations P102L, D178N and E200K have a unique PrPSc glycoform ratio that differs significantly from PrPSc glycoform ratios we have observed in sporadic, iatrogenic and vCJD (Collinge et al., 1996; Hill et al., 2003). Whereas the glycoform profile of PrPSc in P102L cases has been reported by others to be similar to that of type 4 PrPSc seen in vCJD (Furukawa et al., 1998), our findings of a highly statistically significant difference (P < 0.004 for either mono, or un-glycosylated PrPSc fragments) strongly support the hypothesis that vCJD is a distinct human prion strain. Propagation of distinct PrPSc isoforms appears to underlie phenotypic variability in human prion diseases (Collinge et al., 1996; Parchi et al., 1996; Puoti et al., 1999; Gambetti et al., 2003; Hill et al., 2003; Head et al., 2004; Zanusso et al., 2004; Polymenidou et al., 2005). Characteristic human PrPSc fragment sizes and glycoform ratios can be maintained on passage in transgenic mice expressing only human PrP and the propagation of these distinct PrPSc types can be correlated with distinct neuropathological phenotypes (Collinge et al., 1996; Hill et al., 1997; Asante et al., 2002; Wadsworth et al., 2004). These data strongly support the ‘protein-only’ hypothesis of prion infectivity and suggest that prion strain variation is encoded through a combination of PrPSc conformation and glycosylation.

The data presented here suggest that point mutations in PRNP either destabilize non-glycosylated PrP, in turn reducing its relative abundance (Petersen et al., 1996), or directly dictate the stoichiometry and packing order of the three PrP glycoforms into disease-related fibrils or other aggregates. The latter explanation is consistent with a conformational selection model of prion transmission barriers (Collinge, 1999; Collinge, 2001; Hill and Collinge, 2003) that predicts that coding changes in PrP act to specify structural preferences for disease-related PrP isoforms. Immunoprecipitation studies using monoclonal antibodies, which distinguish between PrP glycoforms, are also consistent with incorporation of glycoforms into PrPSc aggregates in strain-specific ratios (Khalili-Shirazi et al., 2005). In contrast to the possible effects of PRNP point mutations, OPRI mutations appear to have little influence in determining the structure of the C-terminal protease-resistant core of PrPSc. Both the glycoform ratio and proteolytic fragment sizes of PrP associated with OPRI mutations are indistinguishable from those of PrPSc seen in classical CJD.

The identification of distinct PrPSc glycoform ratios associated with PRNP point mutations extends the spectrum of PrPSc isoforms seen in human prion disease and reinforces the hypothesis that glycosylation may encode phenotypic information that defines distinct prion strains (Collinge et al., 1996; Lawson et al., 2005; Collinge, 2001). Our findings also support earlier studies (Piccardo et al., 1998, 2001) showing that individual PRNP mutations do not restrict abnormal PrP isoforms to adopting a single pathogenic conformation. Indeed, all of the PrPSc fragment sizes seen in sporadic CJD (Hill et al., 2003) can be observed in PRNP E200K cases. Additionally, the conformational plasticity seen in the N-terminus of type 1 PrPSc in sporadic CJD (Wadsworth et al., 1999; Hill et al., 2003) is similarly observed in PrPSc with the same fragment size in PRNP E200K 129MM cases. Collectively, these data provide an insight into how diverse clinical phenotypes may arise in patients with the same PRNP mutation.

Despite these advances, PrPSc type, as currently defined, cannot satisfactorily explain all of the phenotypic differences seen in inherited prion disease. For example, our data show that cases of D178N FFI and cases of E200K CJD can share the same PrPSc glycoform ratio and propagate the same type 2 PrPSc fragment size. Clearly, further studies are required to investigate other differences in these PrPSc species. In this context it is important to note that we probe PrPSc conformation by looking at accessibility to scissile bonds cleaved by proteinase K at the N-terminus of PrPSc only. Given the site of the PRNP point mutations it seems likely that conformational heterogeneity may exist in the C-terminal structured domain of PrPSc isoforms that are not revealed with proteinase K. Neuropathology associated with specific PRNP mutations
may also be determined by propagation of abnormal isoforms of wild-type PrP (Gabizon et al., 1996; Chen et al., 1997; Silvestrini et al., 1997), and it is also clear that propagation of abnormal PrP isoforms and associated neuropathology may be determined by genetic background (Lloyd et al., 2001, 2004; Asante et al., 2002).

The absence of detectable PrPSc is a consistent feature in inherited prion disease associated with certain PRNP mutations and a more variable feature in others (Collinge, 2001; Kovacs et al., 2002). A possible explanation for some of these findings may relate to sampling variation and in particular the region of brain studied. We examined frontal cortex and therefore with negative samples we cannot exclude the propagation of PrPSc in other brain regions. Nevertheless, sampling variation is unlikely to account for the emerging consensus of similar findings from different laboratories. These collective data indicate that PrPSc isoforms may be generated in inherited prion disease with unique physico-chemical properties, reflected by sensitivity to proteasome K digestion or PrPSc/prion infectivity ratios that are very different from the PrPSc types propagated in sporadic and acquired forms of human prion disease. For example in P102L inherited prion disease, it is now quite clear that patients propagate at least two distinct abnormal PrP conformers. Abnormal PrP conformers generating high molecular mass protease resistant PrP fragments of ~21–30 kDa are not uniformly detected throughout the brain in P102L inherited prion disease and appear to be restricted to areas of the brain showing synaptic PrP deposition and spongiform vacuolation (Parchi et al., 1998; Piccardo et al., 1998). In brain regions in which amyloid plaques are a prominent feature an alternate abnormal PrP isoform appears to predominate that generates smaller protease-resistant PrP fragments of ~8 kDa derived from the central portion of PrP (Parchi et al., 1998; Piccardo et al., 1998) indicating that both the N- and C-terminal residues of this abnormal PrP isoform are digested by proteasome K. Clearly, new experimental methods may be required in order to fully document the spectrum of abnormal PrP isoforms seen in inherited prion disease. In this context it is important to note that not all inherited prion disease may invoke disease through the same mechanism. For example the PRNP A117V mutation gives rise to transmembrane forms of PrP (PrPSm), which have been shown to invoke a neurological disease without generation of PrPSc (Hegde et al., 1998). It is also not presently known whether all inherited prion diseases are transmissible by inoculation.

In summary, we have found that the glycoform ratios of PrPSc in inherited prion disease associated with PRNP point mutations P102L, D178N and E200K are similar to each other, yet significantly different to glycoform ratios of PrPSc observed in sporadic, iatrogenic and vCJD. Since the glycoform ratio of PrPSc associated with PRNP point mutations is significantly different to those arising from PRNP OPRI mutations this suggests that the point mutations may act pathogenically by dictating preferred PrPSc assembly states. Our findings have identified key isolates of inherited prion disease whose transmission properties are now being investigated in appropriate lines of transgenic mice expressing either wild-type or mutated forms of human PrP. These studies will be critical for further understanding the divergent pathogenic mechanisms associated with distinct PRNP mutations.

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Conflict of interest statement

J.C. is a Director and J.C., A.F.H. and J.D.F.W. are shareholders and consultants of D-Gen Limited, an academic spin-out company working in the field of prion disease diagnosis, decontamination and therapeutics.

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