Abnormal prion protein in the pituitary in sporadic and variant Creutzfeldt–Jakob disease

Alexander H. Peden,1 Diane L. Ritchie,¹ Hafsana P. Uddin,1† Andrew F. Dean,2 Kimberley A. F. Schiller,¹ Mark W. Head¹ and James W. Ironside¹

1National Creutzfeldt–Jakob Disease Surveillance Unit (NCJDSU) and Division of Pathology, School of Molecular and Clinical Medicine, University of Edinburgh, Western General Hospital, Edinburgh, UK
2Addenbrooke’s Hospital, Cambridge University Hospitals NHS Foundation Trust, Hills Road, Cambridge, UK

Correspondence
Alexander H. Peden
A.Peden@ed.ac.uk

1National Creutzfeldt–Jakob Disease Surveillance Unit (NCJDSU) and Division of Pathology, School of Molecular and Clinical Medicine, University of Edinburgh, Western General Hospital, Edinburgh, UK
2Addenbrooke’s Hospital, Cambridge University Hospitals NHS Foundation Trust, Hills Road, Cambridge, UK

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By using high-sensitivity Western blotting and immunohistochemistry, pituitary glands from patients with sporadic and variant Creutzfeldt–Jakob disease (sCJD and vCJD, respectively) were analysed for the presence of the protease-resistant form of the prion protein (PrP res ). PrP res was detected in a greater proportion of vCJD pituitaries than sCJD pituitaries and was localized predominantly in the neurohypophysis. PrPres was also detected in a recurrent pituitary adenoma from an sCJD patient. Immunohistochemical analysis showed sparse positive labelling, predominantly in folliculostellate cells, in vCJD and sCJD adenohypophyses. The PrP res glycosylation pattern in the vCJD neurohypophyses showed a predominance of the unglycosylated band, which differed markedly from patterns found in all other vCJD tissues. The presence of PrP res in the pituitary of CJD patients at autopsy suggests that human growth hormone-related iatrogenic CJD may have indeed resulted from infectivity in collected pituitaries rather than necessarily from contamination of pituitary pools by adjacent brain tissue.

To date, there have been 194 deaths worldwide from iatrogenic Creutzfeldt–Jakob disease (iCJD) in patients who had previously received treatment with human growth hormone (hGH) from cadaveric pituitaries, and four deaths similarly attributed to treatment with human gonadotrophin (Brown et al., 2006; Ladogana et al., 2005; Will, 2003). This suggests that pooled stocks of these hormones were contaminated with infectious prions, presumably from the pituitary gland(s) of one or more individuals who were incubating or had died from CJD (Gibbs et al., 1993). This was verified when CJD transmission was achieved in one of 76 lots of hGH used to treat patients in the USA (Gibbs et al., 1993). Despite this, no extensive analyses have yet been carried out on pituitary glands from CJD patients for the presence and localization of the protease-resistant form of the prion protein (PrP res ), which is a marker for the infectious agent in this disease. In this study, the levels of PrP res in the pituitary gland in sporadic and variant CJD (sCJD and vCJD, respectively) were investigated and the localization and Western blot ‘glycoform signature’ of pituitary PrP res were determined.

Pituitary tissue samples taken at autopsy from 11 vCJD patients, nine sCJD patients, including one patient with a recurrent pituitary adenoma, and eight cases of clinically suspected CJD that were given an alternative final pathological diagnosis (included as negative controls) were analysed by Western blotting and/or immunohistochemistry (IHC). All selected cases were of UK origin and had consent for tissue retention and research use. Ethical approval is covered by LREC 2000/4/157 (J. W. I.). The brain from each case had been examined histologically and biochemically and a definite diagnosis of vCJD or sCJD (or non-CJD) was reached by established criteria (Budka et al., 1995; Ironside et al., 2000). The CJD cases were classified according to their PRNP codon 129 genotype (MM, MV or VV) and their brain PrP res molecular subtype (1, 2A or 2B) using the accepted nomenclature (Gambetti et al., 2003; Parchi et al., 1996). The latter is based on Western blot analysis of PrP res in brain following limited digestion with proteinase K, which is used to determine the apparent molecular mass of the unglycosylated PrP fragment and the ratio of PrP glycoforms present. Using this nomenclature, all vCJD cases are MM2B (Head et al., 2004a).

For Western blot analysis, pituitary samples were dissected into the adenohypophysis (anterior lobe) and neurohypophysis (posterior lobe) and homogenized to 10% (w/v) in...
2% Sarkosyl/PBS by using a FastPrep instrument (Qbiogene). In some cases, there was only sufficient tissue available for analysis of the adenohypophysis (Table 1). Samples were then precipitated with sodium phosphotungstic acid (NaPTA), proteinase K-digested and immunoblotted by using the anti-PrP antibody 3F4 (DakoCytomation) as the primary reagent as described previously (Glatzel et al., 2003; Head et al., 2003, 2005; Wadsworth et al., 2001). Western blots were developed by enhanced chemiluminescence (ECL) using either SuperSignal West Femto (Perbio Science) or ECL plus (GE Bioscience). For densitometric analysis, immunoblot images were scanned by using a Bio-Rad GS-800 densitometer and analysed with Quantity One software (Bio-Rad).

IHC was carried out on 5 μm formalin-fixed paraffin-embedded sections of pituitary tissue as described previously (Hilton et al., 2004). PrP was detected by using the anti-PrP antibodies 6H4 (Prionics) and 12F10 (Immunodiagnostics Systems) and visualized by using the CSA amplification system (DakoCytomation) (Sabattini et al., 1998). Selected sections were also stained with antibodies to S100 protein and glial fibrillary acidic protein (GFAP; DakoCytomation).

By using NaPTA precipitation and Western blotting, PrPres was detected in the pituitaries from some, but not all, vCJD and sCJD patients. PrPres was detected more readily in vCJD pituitaries, with five out of eight pituitaries testing positive (Table 1). In three vCJD patients (V8, V11 and V17), where tissue was available from both lobes of the pituitary, PrPres was found predominantly in the neurohypophysis (Fig. 1). PrPres was less detectable in sCJD pituitaries, with three pituitaries testing positive out of nine tested, including one patient, S28, who had a recurrent null-cell pituitary adenoma at autopsy (Table 1). PrPres was detected in the recurrent adenoma, but was present at a higher level in the neurohypophysis (Fig. 1). No PrPres was detected in the non-CJD pituitaries (data not shown). As reported previously (Wadsworth et al., 2001), NaPTA precipitation prior to proteinase K digestion caused a slight upward shift in the mobility of PrPres (Fig. 1, lower panel).

The signals for the vCJD neurohypophysis samples (Fig. 1, upper panels) obtained using SuperSignal West Femto as the ECL detection reagent were too intense to allow determination of the ratio for the di-, mono- and unglycosylated (upper, middle and lower) PrPres bands. Therefore, the immunobLOTS were immediately washed and retreated with

<table>
<thead>
<tr>
<th>Patient</th>
<th>Western blot analysis</th>
<th>IHC analysis</th>
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<tbody>
<tr>
<td></td>
<td>Adenohypophysis</td>
<td>Neurohypophysis</td>
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<tr>
<td>vCJD</td>
<td></td>
<td></td>
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<tr>
<td>V3</td>
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<tr>
<td>V4</td>
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<tr>
<td>V8</td>
<td>–</td>
<td>+ (1/9)</td>
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<tr>
<td>V10</td>
<td>–</td>
<td>–</td>
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<tr>
<td>V11</td>
<td>–</td>
<td>+ (1/11)</td>
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<tr>
<td>V13</td>
<td>+ (1/26)</td>
<td>NT</td>
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<td>V14</td>
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<td>V15</td>
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<td>V18</td>
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<tr>
<td>sCJD</td>
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<tr>
<td>S13</td>
<td>+ (1/1138)</td>
<td>NT</td>
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<tr>
<td>S14</td>
<td>+ (1/1173)</td>
<td>NT</td>
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<tr>
<td>S20</td>
<td>–</td>
<td>NT</td>
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<td>S26</td>
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<tr>
<td>S28*</td>
<td>+ (1/654) (adenomatous tissue)</td>
<td>+ (1/65)</td>
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*Patient with a recurrent pituitary adenoma.

Table 1. Summary of Western blot and IHC analysis of vCJD and sCJD pituitaries

PrPres levels per unit mass of tissue compared with standard vCJD brain frontal cortex, as determined by densitometric analysis of Western blots, are given in parentheses. +, Positive; –, negative; NT, not tested.
the ECL plus reagent, which gives a less intense signal. By using this method, the PrP\textsuperscript{res} glycosylation pattern for the neurohypophyses of the vCJD patients and the neurohypophysis of sCJD patient S28 with a recurrent pituitary adenoma showed a predominance of the unglycosylated band (e.g. Fig. 1, patients V17 and S28, lower panels).

Smaller volumes of neurohypophysis extracts from the vCJD patients (0.5–2.3 mg tissue) were proteinase K-treated without NaPTA precipitation and immunoblotted. A predominance of the unglycosylated PrP\textsuperscript{res} band for the vCJD neurohypophysis was observed again, indicating that this pattern was not an artefact of NaPTA precipitation (Fig. 1, patients V8 and V17, lower panel). The glycoform ratios (di- : mono- : un-) for the neurohypophyses of vCJD patients V8 and V17 determined by densitometry were 20 : 31 : 49 % and 31 : 30 : 39 %, respectively, which are markedly different from the 48 : 38 : 14 % ratio determined for vCJD brain (Collinge et al., 1996; Head et al., 2004a). The glycoform ratio determined for PrP\textsuperscript{res} in the neurohypophysis of sCJD patient S28 also showed a predominance of the unglycosylated form (7 : 37 : 56 %) when compared with brain frontal cortex from the same case (23 : 37 : 40 %) (Fig. 1). Interestingly, this was not observed for the adenoma from this case and the adenohypophysis from sCJD case S13, where the diglycosylated form of PrP\textsuperscript{res} was predominant.

The PrP\textsuperscript{res} levels in the pituitary samples were determined by densitometry by reference to a dilution series of brain frontal cortex (5–500 μg tissue) from a single vCJD patient run on the same immunoblot (data not shown). The PrP\textsuperscript{res} levels per unit mass of tissue in the vCJD neurohypophyses were approximately one-tenth of the level in standard vCJD brain (Table 1). PrP\textsuperscript{res} levels in the vCJD adenohypophyses were considerably lower (1/25 to 1/500 compared with vCJD brain). The three positive sCJD adenohypophysis samples contained levels of PrP\textsuperscript{res} that were all lower than 1/600 compared with the same vCJD brain standard (Table 1). However, the PrP\textsuperscript{res} level in the neurohypophysis from sCJD case S28 was comparable to that in vCJD neurohypophyses, being 1/65 the level in vCJD brain.

The clinical durations of vCJD in the five patients testing positive for pituitary PrP\textsuperscript{res} by Western blotting were not markedly different from the three cases that were negative, all falling in the range of 7–18 months, which is typical for vCJD. Interestingly, two of the three sCJD patients who were positive for pituitary PrP\textsuperscript{res} (S14 and S28) had atypically long clinical durations (21 and 15 months, respectively), whereas S13 had a clinical duration of 4 months, the median duration for sCJD (Gambetti et al., 2003). Two of the three positive sCJD patients (S14 and S28) had PRNP codon 129 genotypes and brain PrP\textsuperscript{res} subtypes that are less common for sCJD (MV2A and MV1, respectively), although S13 had MM1, which is the most common type (Gambetti et al., 2003; Parchi et al., 1996).

IHC analysis showed widespread granular PrP\textsuperscript{res} deposition in the pituicytes of the neurohypophysis, which are specialized glial cells of the neurohypophysis (Table 1), in all vCJD cases (Fig. 2a). IHC analysis of the pituitary adenoma from sCJD case S28 showed the deposition of PrP\textsuperscript{res} within occasional neoplastic cells (Fig. 2b). IHC analysis of the neurohypophysis from this case indicated widespread PrP\textsuperscript{res} deposition similar to that seen for the other sCJD and vCJD patients (data not shown). Comparatively scanty PrP\textsuperscript{res} deposits were seen the adenohypophysis (Fig. 2c). Similar findings were present in sCJD pituitaries, but both the distribution and intensity of labelling were weaker overall, with one negative case (Table 1). The scarce deposits in the adenohypophysis were mainly in folliculostellate (FS) cells, which also stained positively with antibodies to S100 protein (Fig. 2d) and...
GFAP (data not shown) (Fauquier et al., 2002). FS cells are specialized, non-secretory cells of the adenohypophysis that are possibly related to glial cells, although their precise function is unknown. Occasional glandular cells stained weakly for PrP, but no specific involvement of any particular cell type was found. No specific labelling was found in non-CJD pituitary controls.

It has been shown here that PrPres is present in the pituitary of CJD patients at autopsy, suggesting that growth hormone-related iCJD may have resulted from infectivity in collected pituitaries rather than necessarily from contamination of pituitary pools by adjacent brain tissue. By using NaPTA precipitation and Western blotting, more vCJD pituitary samples tested positive than sCJD pituitaries. In vCJD, PrPres was mainly detected in the neurohypophysis, which has direct connections with the hypothalamus, suggesting that PrPres may have been deposited there as a result of centrifugal spread from the brain. Assuming the latter to be true, the higher levels of PrPres detected in vCJD neurohyphophyses may be due to the longer clinical duration of vCJD compared with sCJD. Given the marked deposition of PrPres in many of the vCJD pituitaries examined, it is interesting that no clinical manifestations of endocrine dysfunction have been reported in vCJD patients. However, it should be noted that there is evidence for hypercortisolism in sheep with scrapie and one explanation for this was disease-induced damage to the pituitary (Gayrard et al., 2000).

The possibility that the low levels of PrPres detected in the vCJD adenohyphophyses by Western blotting may be due to cross-contamination of samples with small amounts of tissue from the neurohyphophysis cannot be excluded. However, IHC indicated scarce but clearly identifiable PrPres deposits predominantly in FS cells, which also stained with S100- and GFAP-specific antibodies. Occasional glandular cells were also labelled for PrP, but with a weaker intensity not confined to any specific cell type.

The predominance of the unglycosylated band for PrPres from vCJD patient neurohyphophyses (Fig. 1) contrasts with the distinctive predominance of the diglycosylated form that is used as a marker for vCJD, which is seen in nearly all other tissues examined from vCJD patients (Head et al., 2004b; Wadsworth et al., 2001) and from a case of preclinical vCJD infection (Peden et al., 2004). Exceptions to this are vCJD retina, where the three PrPres bands appear to be approximately equal in intensity (Head et al., 2003; Wadsworth et al., 2001), and vCJD trigeminal ganglion, where the monoglycosylated and unglycosylated bands are dominant (Head et al., 2003), which is comparable to type 2A sCJD (Parchi et al., 1996).

A predominance of the unglycosylated PrPres band was also seen in the neurohyphophysis of sCJD patient S28, who had a recurrent pituitary adenoma, although prevalence of the diglycosylated band was seen in the adenoma of this case and the non-neoplastic adenohypophysis of another sCJD case S13, which is reminiscent of the pattern seen in vCJD brain. Our analysis of pituitaries indicates that the cell types in which PrPres accumulates can have an overriding effect on strain-associated biochemical properties, thus placing limitations on, for example, the ‘glycoform signature’ as a reliable marker of the bovine spongiform encephalopathy/vCJD agent.

Patient S28 was the only sCJD patient who showed high levels of PrPres in the neurohyphophysis. Even though the pituitary adenoma in this patient had recurred, the PrPres levels detected in the adenoma were higher than in other non-neoplastic sCJD adenohyphophyses (Table 1). This suggests that PrPres had accumulated within the adenoma and IHC analysis of the adenoma indicated that PrPres had

![Fig. 2.](a) Paraffin section stained with anti-PrP antibody 12F10 showing granular PrP deposition in pituicytes in the neurohypophysis from vCJD patient V13. Bar, 50 μm. (b) Section showing the pituitary adenoma from sCJD patient S28, stained with anti-PrP antibody 12F10, showing PrP deposition in FS cells. Bar, 25 μm. (c) Section of the adenohypophysis from vCJD patient V15, stained with anti-PrP antibody 12F10, showing PrP deposition in FS cells. Bar, 100 μm. (d) A section serial to (c) labelled with the S100 antibody to demonstrate FS cells. Bar, 100 μm. All sections were counterstained with haematoxylin.)
deposited within occasional neoplastic cells (Fig. 2b). These findings are consistent with the high amounts of PrP^res detected in neoplastic lymphoreticular tissues of vCJD-infected mice, which suggested that rapidly growing lymphoreticular tumours accumulate PrP^res at a high rate (Cervenakova et al., 2006).

The occurrence of cases of iCJD associated with hGH therapy suggested that the infectious agent was present in the stocks of hGH used to treat patients in the affected countries, principally France, the USA and the UK (Brown et al., 2006). The source of contamination in pooled pituitaries is most likely to have come from individuals with sCJD, as this is the most common form of CJD (Ladogana et al., 2005). The heterogeneous, low-level deposition of PrP^res that was observed in the sCJD pituitaries is consistent with the scarce amounts of infectivity detected in hGH batches that had been used to treat patients in the USA who subsequently contracted iCJD (Gibbs et al., 1993). Although the specific risk associated with the use of human pituitary-derived hormones is now avoided by using biosynthetic compounds, this study is directly relevant to ongoing risk assessments for the potential for the transmission of CJD via surgical instruments.

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References


