

Musculoskeletal Pathology

Detection and Localization of PrP^{Sc} in the Skeletal Muscle of Patients with Variant, Iatrogenic, and Sporadic Forms of Creutzfeldt-Jakob Disease

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Variant Creutzfeldt-Jakob disease (vCJD) differs from other human prion diseases in that the pathogenic prion protein PrP^{Sc} can be detected to a greater extent at extraneuronal sites throughout the body, principally within lymphoid tissues. However, a recent study using a high-sensitivity Western blotting technique revealed low levels of PrP^{Sc} in skeletal muscle from a quarter of Swiss patients with sporadic CJD (sCJD). This posed the question of whether PrP^{Sc} in muscle could also be detected in vCJD, sCJD, and iatrogenic (iCJD) patients from other populations. Therefore, we have used the same high-sensitivity Western blotting technique, in combination with paraffin-embedded tissue blotting, to screen for PrP^{Sc} in muscle tissue specimens taken at autopsy from 49 CJD patients in the United Kingdom. These techniques identified muscle PrP^{Sc} in 8 of 17 vCJD, 7 of 26 sCJD, and 2 of 5 iCJD patients. Paraffin-embedded tissue blotting analysis showed PrP^{Sc} in skeletal muscle in localized anatomical structures that had the morphological and immunohistochemical characteristics of nerve fibers. The detection of PrP^{Sc} in muscle tissue from all forms of CJD indicates the possible presence of infectivity in these tissues, suggesting important implications for assessing the potential risk of iatrogenic spread via contaminated surgical instruments. (*Am J Pathol* 2006, 168:927-935; DOI: 10.2353/ajpath.2006.050788)

Creutzfeldt-Jakob disease (CJD) is a member of the human prion diseases or transmissible spongiform encephalopathies, a group of fatal degenerative diseases of the central nervous system (CNS). A key event in the patho-

genesis of prion diseases is the conversion of the cellular prion protein PrP^C to the abnormal disease-associated form PrP^{Sc}. According to the prion hypothesis, PrP^{Sc} is the principle or sole component of the infectious agent, and the accumulation of PrP^{Sc} within the CNS has been proposed to be the primary event leading to neurodegeneration.¹

Prion diseases occur in idiopathic, inherited, and acquired forms. The most common human prion disease is sporadic CJD (sCJD), which occurs worldwide with a frequency of approximately one per million of the population per annum.²⁻⁴ The cause of this disease is unknown, although a random stochastic event resulting in a conversion of PrP^C to PrP^{Sc} and the subsequent propagation of this process is one possibility.⁵ CJD can also be acquired by iatrogenic transmission (iCJD) as a result of certain neurosurgical procedures, dura mater or corneal transplantation, and through treatment with cadaveric human growth hormone as reviewed by Brown and colleagues.⁶ A novel acquired human prion disease, variant Creutzfeldt-Jakob disease (vCJD), is thought to result from oral exposure to the bovine spongiform encephalopathy agent.⁷ vCJD is clearly distinct from other forms of CJD in its neurological, neuropathological, and biochemical phenotype.

PrP^{Sc} differs from PrP^C in its relative resistance to proteases and is often referred to as PrP^{res} after partial proteolytic degradation. Although PrP^{Sc} accumulation occurs primarily in the brain, PrP^{res} has also been detected in the peripheral tissues of CJD patients.⁸⁻¹⁰ This is particularly the case for vCJD in which PrP^{res} is readily detected in the lymphoid tissues including, spleen, tonsil, lymph nodes, and Peyer's patches using standard Western blotting and immunohistochemistry techniques.^{9,10} In

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contrast, in sCJD PrP^{res} accumulation (and by implication infectivity) was thought to be primarily confined to the CNS.^{9–11} However, earlier transmission studies had demonstrated infectivity in extraneural tissues, including spleen, lung, liver, and kidney, but not skeletal muscle or peripheral nerve.¹² Also, there was always the possibility that the detection of low levels of PrP^{res} in peripheral organs in all forms of CJD may be limited by the sensitivity of the assays used. This was highlighted recently by Glatzel and colleagues⁸ who were able to detect PrP^{res} in autopsy specimens of spleen and muscle in a proportion of sCJD patients in Switzerland using a high-sensitivity Western blotting technique involving selective precipitation of PrP^{res} with sodium phosphotungstic acid (NaPTA).

The detection of PrP^{res} in the muscle of Swiss sCJD patients presents the worrying prospect of the transmission of prion infection via instruments used in ordinary surgical procedures. Indeed, a collaborative case control study performed on sCJD patients in Europe showed that a past history of surgery was a risk factor.¹³ The study by Glatzel and colleagues⁸ requires confirmation in sCJD patients from other populations. The study was purely biochemical in nature and did not investigate the localization of PrP^{res} within the muscle tissue. Lastly, the work of Glatzel and colleagues⁸ raises the question of whether PrP^{res} is present in the muscle of patients with other forms of CJD, most notably vCJD, where the extent of peripheral PrP^{res} tends to be greater.^{9,10} Because of the oral route of infection in vCJD and the presumed long incubation period,¹⁴ infectious prions may be present in the peripheral tissues for a considerable period of time before the onset of clinical symptoms. For this reason vCJD may pose an increased risk for the iatrogenic transmission of the disease. This risk had been highlighted recently by two cases of the secondary transmission of vCJD infection by blood transfusion from donors who only developed vCJD after donation.^{15,16}

To address the above issues, we have used the same high-sensitivity NaPTA precipitation/Western blotting technique to screen skeletal and heart muscle samples from UK patients with vCJD, iCJD, as well as sCJD. We show that PrP^{res} is indeed present in a significant proportion of autopsy skeletal muscle samples taken from vCJD, iCJD, and sCJD patients, and we go on to use the paraffin-embedded tissue (PET) blotting technique to determine the localization of PrP^{res} within human muscle tissue.

Materials and Methods

Cases and Tissue Specimens

Seventeen vCJD patients, twenty-six sCJD cases, and five iCJD cases (associated with human growth hormone therapy) were analyzed in this study by a combination of Western blotting and PET blotting. Twelve cases of clinically suspected CJD that were given an alternative final pathological diagnosis were included as negative controls because they would be expected to lack PrP^{res} in the brain and peripheral tissues. Cases were selected for

this study on the basis of the availability of fixed and frozen tissue specimens retained at autopsy and the existence of consent for tissue retention and research use. Ethical approval for the acquisition and use of autopsy material for research on transmissible spongiform encephalopathies in the National CJD Surveillance Unit brain bank is covered by LREC 2000/4/157 (Prof. J.W. Ironside). All autopsy cases were of UK origin. The brain from each case had previously been examined histologically and biochemically, and a definite diagnosis of variant, sporadic, or iatrogenic CJD (or non-CJD) had been reached by established criteria.^{17,18} The protease-resistant prion protein (PrP^{res}) isotype found in brain was classified as type 1, 2A, or 2B as previously described according to the nomenclature of Parchi and colleagues.^{19–22} The polymorphic status of codon 129 of the prion protein gene *PRNP* of each case was determined by restriction fragment length polymorphism.²²

Western Blot Analysis

The skeletal muscle taken at autopsy was from quadriceps, sternomastoid, or intercostal muscles. Frozen skeletal and cardiac muscle tissues from CJD and non-CJD control patients were analyzed by the high-sensitivity Western blotting protocol described by Glatzel and colleagues⁸ and Wadsworth and colleagues¹⁰ previously, with a few modifications. Briefly, 10% w/v extracts were made of 65- to 100-mg samples of tissue by homogenizing in an appropriate volume of ice-cold 2% sarkosyl/phosphate-buffered saline (PBS), pH 7.4, using the Fast-Prep instrument (Qbiogene, Cambridge, UK). The samples were then cleared by centrifugation at 5200 × *g* for 5 minutes at 4°C. Samples (0.5 ml) of the cleared lysates were diluted with a further 0.5 ml of 2% sarkosyl/PBS and incubated for 10 minutes at 37°C. Benzonase (Sigma, Poole, UK) and MgCl₂ were added at final concentrations of 50 U/ml and 1 mmol/L, respectively, and incubation at 37°C was continued for a further 30 minutes. Eighty-one μl of a stock solution of 4% w/v NaPTA and 170 mmol/L MgCl₂, pH 7.4, was added (final concentration of NaPTA, 0.3% w/v), and precipitation was allowed to occur for 30 minutes at 37°C. The samples were centrifuged at 20,800 × *g* for 30 minutes at 37°C. The resultant supernatant was discarded and the pellets were resuspended in 20 μl of 0.1% w/v sarkosyl in PBS, pH 7.4, and digested with 50 μg/ml proteinase K for 30 minutes. Digestion was terminated by the addition of 1 mmol/L PefaBloc SC (Roche, Lewes, UK).

Electrophoresis was performed using the NuPAGE Novex gel system (Invitrogen, Paisley, UK). Before electrophoresis, NuPAGE LDS sample buffer was added to each of the samples to a final concentration of 1×. The samples were boiled for 10 minutes and separated on 10% Bis-Tris NuPAGE gels. The separated proteins were then transferred onto immunoblot polyvinylidene difluoride membrane (Bio-Rad, Hertfordshire, UK). For immunodetection, the anti-PrP monoclonal antibody 3F4 (DakoCytomation, Cambridgeshire, UK) was used at a final concentration of 50 ng/ml IgG for 1 hour. Horseradish

peroxidase-conjugated anti-mouse IgG F(ab')₂ fragment (Amersham Biosciences, Amersham, UK) was used at a dilution of 1 in 40,000 for 1 hour. The detection reagent used was SuperSignal West fempto maximum sensitivity substrate (Pierce, Rockford, IL). The immunoblots were exposed to HyperFilm ECL (Amersham Biosciences) for periods of 10 seconds to 30 minutes. The molecular weight of PrP^{res} was estimated by reference to IgG-binding MagicMark XP Western protein standards (Invitrogen), and standard PrP^{res} samples from vCJD brain were run on all blots throughout the study. These standards were prepared by PK digestion using the conventional method described previously.^{18,23}

Criteria for Assigning Positives

Frozen muscle samples from nine non-CJD neurological patients were available for use as negative controls in the Western blots in this study. As a positive control in these experiments, 10 μ l of 1% w/v vCJD (type MM2B) brain homogenate was diluted into 0.5 ml of 10% w/v muscle tissue homogenate from one of the non-CJD control patients. This spiked muscle homogenate was then diluted with a further 0.5 ml of 2% sarkosyl as described above. Precipitation with NaPTA and PK digestion were performed as described for the test samples. Samples of CJD patient muscle tissue were assessed by comparison with positive and negative control samples run either on the same gel or on a separate gel run in parallel at the same time using exactly the same conditions.

Our laboratory and others have shown tissue-specific effects on the apparent glycosylation profile of PrP^{res} that can lead to an underrepresentation of one of the three characteristic bands observed on the Western blots.^{9,10} Therefore, the following criteria were established before interpreting the results. A positive result was assigned if three or at least two bands were observed to co-migrate with any of the corresponding PrP^{res} bands in the positive control and no bands or smears were seen in the lane containing non-CJD control muscle sample, after maximum exposure to HyperFilm ECL. A negative result was assigned if less than two bands were present in the correct region in the lane containing the test sample on a blot where the positive control was detected.

Immunohistochemistry and PET Blotting

The localization of PrP^{res} in paraffin sections of cardiac and skeletal muscles was investigated using the highly sensitive PET blot method.^{24,25} To establish the cellular location of PrP^{res} deposits, conventional immunohistochemistry was performed to compare the localization of a number of cellular markers with the distribution of PrP^{res}. Serial 5- μ m sections from both formalin-fixed and periodate-lysine paraformaldehyde-fixed, formic acid-treated sections were mounted on Superfrost plus slides (VWR, Poole, UK) for immunohistochemistry and on 0.45- μ m nitrocellulose membrane for PET blot analysis. Slides and membranes were incubated overnight at 55°C before use.

PET Blotting

PET blot analysis was performed as described by us previously²⁴ using a modified version of the method of Schulz-Schaeffer and colleagues²⁵. Briefly, sections mounted on nitrocellulose were dewaxed before an overnight digestion in 25 μ g/ml of proteinase K. Membranes were washed in Tris-buffered saline containing 0.1% Tween-20 before treatment with 3 mol/L guanidine isothiocyanate for 10 minutes. After a further wash the membranes were blocked with casein and incubated for 2 hours with 3F4 antibody (1:500 dilution). Labeling was completed using a Vectastain ABC-AmP detection system (Vector Laboratories, Peterborough, UK) and visualized using the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate chromogen system. Sections from sCJD brain were included with each PET blot run as a positive control for the primary antibody. Labeling was observed and photographed using a stereomicroscope.

Immunohistochemistry

Investigations on the cellular location of PrP^{res} in muscle tissue were performed using antibodies raised against neurofilament protein, neuron-specific enolase, synaptophysin, and S-100 protein (all from DakoCytomation). Before immunolabeling sections were dewaxed and immersed in picric acid to remove formalin pigment. After washing in water all sections were pretreated by microwaving in 10 mmol/L citrate buffer, pH 6.0, for 15 minutes. Immunolabeling was then completed using the Envision Plus HRP kit (DakoCytomation) for the anti-neuron-specific enolase antibody of Vectastain Elite ABC kit (Vector Laboratories) for the other antibodies. Staining was visualized using diaminobenzidine.

Results

Western Blotting

We applied our modified Western blotting protocol to assess muscle tissue samples from a total of 49 CJD patients and 9 non-CJD neurological disease patients.

Patients with vCJD

Seventeen patients with a definite diagnosis of vCJD (numbered V1 to V17) were analyzed for PrP^{res} in their skeletal muscle, and eight positives were identified (Figure 1) using the criteria described above. The immunoblot for patient V9 shows that samples taken from these patients contained varying amounts of PrP^{res} (Figure 1, top). Samples assigned as positive from the remaining seven vCJD patients are shown together in the bottom panel of Figure 1. Only a proportion of samples taken from these patients contained detectable PrP^{res} (Table 1), suggesting that PrP^{res} has a heterogeneous distribution within the muscle tissue. The samples that were positive contained PrP^{res} at levels approximately equal to the positive control, which contained 100 μ g of vCJD brain homogenate. Because each sample is the NaPTA

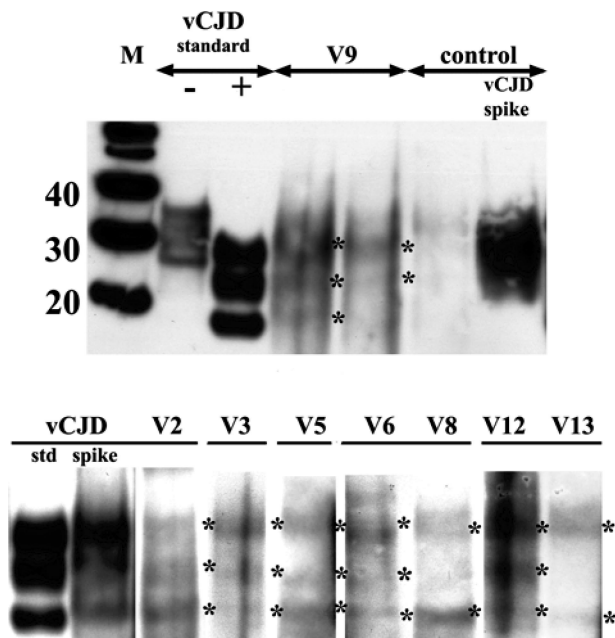


Figure 1. Western blots of vCJD patient muscle samples positive for PrP^{res}. Samples of muscle homogenate equivalent to 50 mg wet weight of tissue were precipitated with NaPTA, digested with PK, and immunoblotted with anti-PrP antibody 3F4 as the primary reagent, as described in the Materials and Methods section. **Top:** A typical immunoblot for assessing CJD patient muscle for the presence of PrP^{res}. Two muscle samples from the vCJD patient V9 are compared with muscle from non-CJD neurological disease control patient, one of which has been spiked with 100 μg of vCJD brain homogenate. The bands that we infer to be PrP^{res} are indicated with **asterisks**. These precipitates were run in parallel with vCJD brain standards before (-) and after (+) PK digestion. The molecular weights in kd of the MagicMark XP standards (M) are indicated on the **left**. **Bottom:** A composite image taken from six separate immunoblots showing muscle samples from the seven other vCJD patients assigned as positive by Western blotting, aligned with vCJD brain standard (100 μg). The gaps in the upper bar indicate where the separate immunoblots have been aligned.

precipitate from 50 mg of wet weight of tissue, we estimate that the levels of PrP^{res} in these positive muscle samples were ~1:500 that of vCJD brain.

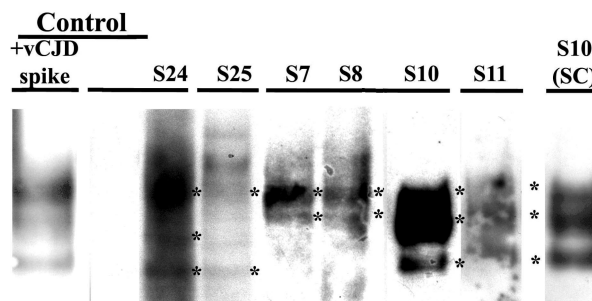


Figure 2. Western blots of sCJD patient muscle samples positive for PrP^{res}. Samples of muscle from six patients assigned as positive are compared with muscle from a non-CJD neurological control patient and vCJD control brain homogenate (100 μg) diluted into non-CJD control muscle homogenate. Two of the patients, S7 and S10, were also positive by PET blotting (see Figure 4). As in Figure 1, the bands that we infer to be PrP^{res} are indicated with **asterisks**. The gaps in the upper bar indicate where separate blots have been aligned. For comparison, the PrP^{res} signal obtained from spinal cord from patient S10 after NaPTA precipitation and Western blotting is shown in the final lane.

Patients with sCJD

Using this same Western blotting technique, 6 of 26 patients with definite sCJD were identified as being positive for skeletal muscle PrP^{res} (Figure 2). Two of these patients, S7 and S10, were also shown to be positive by PET blotting (see below). Patient S10 had a particularly strong signal for PrP^{res} in all three samples taken. Interestingly, the unglycosylated PrP^{res} fragment was under-represented in the muscle from patient S10 when compared with PrP^{res} from the CNS (spinal cord) of this patient (Figure 2). The bands identified as being PrP^{res} in the other positive samples were often diffuse. However, the signals obtained were distinct from the results obtained from an analysis of skeletal muscle from nine patients with non-CJD neurological disease. A total of 22 muscle samples were analyzed from this cohort of nine patients, and no bands were observed even on extended

Table 1. Pathological, Genetic, and Clinical Profiles of CJD Patients with Muscle Shown to Be Positive for PrP^{res} by Western Blotting (WB) and PET Blotting

Case	WB	PET	Codon 129	Brain PrP ^{res} type
vCJD patients (17 analyzed)				
V2	(1/4)	(0/1)	MM	2B
V5	(3/7)	(0/1)	MM	2B
V6	(1/2)	(0/1)	MM	2B
V8	(1/7)	(0/1)	MM	2B
V9	(2/4)	(0/1)	MM	2B
V12	(1/13)	(0/1)	MM	2B
V13	(1/3)	(0/1)	MM	2B
sCJD patients (26 analyzed)				
S7	(1/2)	(1/1)	MV	2A (+1)
S8	(1/2)	(0/1)	MM	1 + 2A
S10	(3/3)	(1/1)	MV	1
S11	(2/7)	(0/1)	MM	1
S14	(0/3)	(1/1)	MV	2A
S24	(1/2)	(0/1)	MM	1
S25	(1/2)	(0/1)	MV	2A
iCJD patients (5 analyzed)				
I4	(4/5)	(0/1)	VV	2A
I5	(0/3)	(1/1)	MV	2A

The number of samples tested (no. positive for PrP^{Sc}/total) is indicated in parentheses. The positive scores are indicated in bold type.

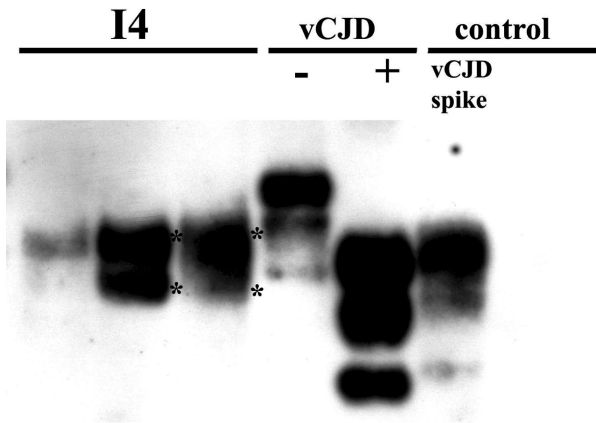


Figure 3. Western blots of iCJD patient muscle samples positive for PrP^{res}. Three samples of muscle from an iCJD patient (I4) are compared with 100 μ g of vCJD brain homogenate diluted in non-CJD control muscle homogenate, NaPTA precipitated, and digested with PK (vCJD spike). Also shown are samples of vCJD brain homogenate before (-) and after (+) digestion with PK (50 μ g and 200 μ g, respectively) without NaPTA precipitation. Non-spiked, non-CJD muscle homogenate, digested with PK, is shown in the last lane as a negative control. As noted previously¹⁰ there is a slight upward shift in the mobility of PrP^{res} after NaPTA precipitation (vCJD spike lane).

exposures, as exemplified by the control samples shown in Figures 1, 2, and 3.

Patients with iCJD

Frozen skeletal muscle tissue from five patients with human growth hormone-associated iCJD were available for analysis by Western blotting. One of these patients (I4) was positive for PrP^{res} for four of five muscle samples taken (Figure 3). Interestingly, the unglycosylated fragment was below the limit of detection in all samples taken. However, using the criteria described above we were able to assign a positive result as two visible bands co-migrated with bands corresponding to the di- and monoglycosylated forms of PrP^{res} in the spiked positive control, which was the NaPTA precipitate from 100 μ g of vCJD (PrP^{res} type MM2B) brain extract diluted in 50 mg of non-CJD control muscle homogenate (Figure 3). As shown in Figure 3, PrP^{res} obtained after NaPTA precipitation and PK digestion in the spiked positive control (vCJD, spike) consistently had a higher apparent molecular weight than PrP^{res} from vCJD extract prepared and PK-digested without prior NaPTA precipitation (vCJD, +). As shown previously NaPTA causes this effect when applied to PrP^{Sc} before proteolysis by altering the PK cleavage site, resulting in the generation of larger PrP^{res} fragments.¹⁰ Because the spiked positive control replicates the conditions used for the NaPTA precipitation and PK digestion of the test samples, the bands obtained for this control should be used for evaluating bands obtained in the control samples. The absence of a positive score for one of the five samples from patient I4 (Figure 3) again implies a heterogeneous distribution of PrP^{res} in the muscle tissue.

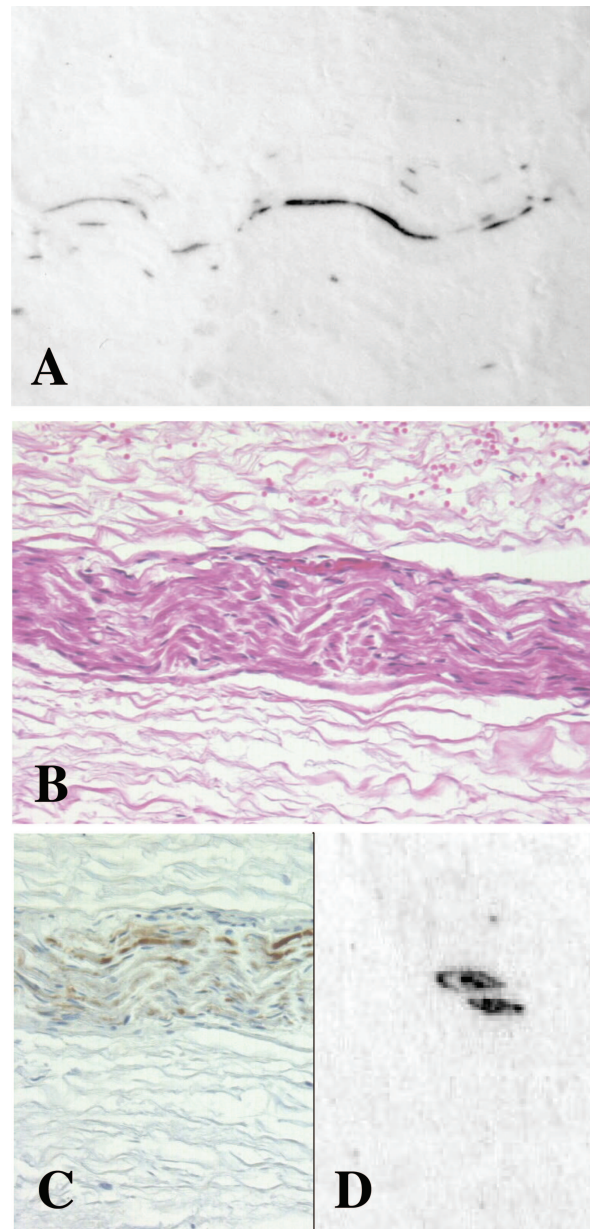


Figure 4. PET blot and immunohistochemical analysis of PrP in sections of sCJD skeletal muscle. **A:** PET blot preparation showing labeling of PrP^{res} in longitudinal nerve fibers within a muscle sample from sCJD patient S10. **B:** Paraffin section adjacent to **A** showing the presence of a nerve fiber bundle in the site of the PrP^{res} labeling (H&E). **C:** Paraffin section adjacent to **A** showing a similar pattern of staining in large diameter axons to the positive labeling for PrP^{res} after immunocytochemistry for neurofilament protein. **D:** PET blot preparation showing labeling of PrP^{res} in an oblique cross-section of large diameter axons within a muscle sample from sCJD patient S7. Original magnifications: $\times 200$ (**A-C**); $\times 400$ (**D**).

Localization of PrP^{res} in Muscle Tissue

PET Blot Analysis of CJD Patients

To investigate the muscle tissue heterogeneity we have used PET blotting to localize PrP^{Sc} in sections of muscle tissue.²⁴ Fixed tissue from 17 vCJD, 22 sCJD, three iCJD, and 11 non-CJD cases in our cohort were available for PET blot analysis using the anti-PrP antibody 3F4. Of the 22 sCJD patients analyzed, three (S7, S10,

and S14) were found to be positive for PrP^{res} (examples shown in Figure 4, A and D). Two of these patients (S7 and S10) had previously been found to be positive by Western blotting (Figure 1). Therefore, this analysis brought the total number of sCJD patients positive for skeletal muscle PrP^{res} to seven (of 26). PET blotting also showed PrP^{res} immunoreactivity in another of the three iCJD patients analyzed. None of the 17 vCJD patients analyzed were positive using this technique, and no labeling was observed in the 11 non-CJD control patients (data not shown). For all four positive samples, PrP^{res} labeling occurred in a linear pattern with a distribution and morphology consistent with nerve fibers (see below). No labeling of muscle fibers, motor end plates, or muscle spindles was observed.

Immunohistochemistry

To further investigate the localization of PrP^{res} at higher resolution within the muscle tissue, further immunohistochemistry analysis was performed on adjacent skeletal muscle sections from the four cases shown to be positive by PET blotting. Antibodies to S100 protein were used to label Schwann cells, and antibodies to synaptophysin, neurofilament protein, and neuron-specific enolase were used to label axons in nerve fibers within the tissue (Figure 4C). In muscle sections from all three sCJD patients (S7, S10, and S14) and the iCJD patient (I5) the pattern of immunoreactivity on these sections closely resembled the pattern seen for PrP^{res} on PET blots of adjacent sections (Figure 4, A and D), with most co-localization seen in small nerve fiber bundles.

Pathological, Genetic, and Clinical Profiles of CJD Patients with PrP^{res}-Positive Skeletal Muscle

The pathological, genetic, and clinical data for the patients positive for muscle PrP^{res} were analyzed against the data for all of the CJD patients in this study. The data on the *PRNP* codon 129 genotype and brain PrP^{res} isotype combinations and duration of illness of the vCJD, sCJD, and iCJD patients identified as positive for PrP^{res} in muscle are shown in Table 1. The clinical durations of all of the sCJD and vCJD patients in this study are presented as box and whisker plots in Figure 5.

The positive sCJD patients (indicated by asterisks) tended to have longer clinical durations; five of the seven patients identified as positive had durations of illness longer than 4 months, the median for this data set (Figure 5) and for sCJD in general.²⁶ Two of the positive sCJD patients were outliers with clinical durations of 21 and 54 months. This may suggest a positive correlation between the duration of illness and the detection of PrP^{res} in muscle, although the numbers of patients analyzed in this study is insufficient for firm conclusions to be drawn. No such correlation was seen within the vCJD group (Figure 5). However, vCJD patients on average have significantly longer clinical durations (median, 14 months¹⁴), and a

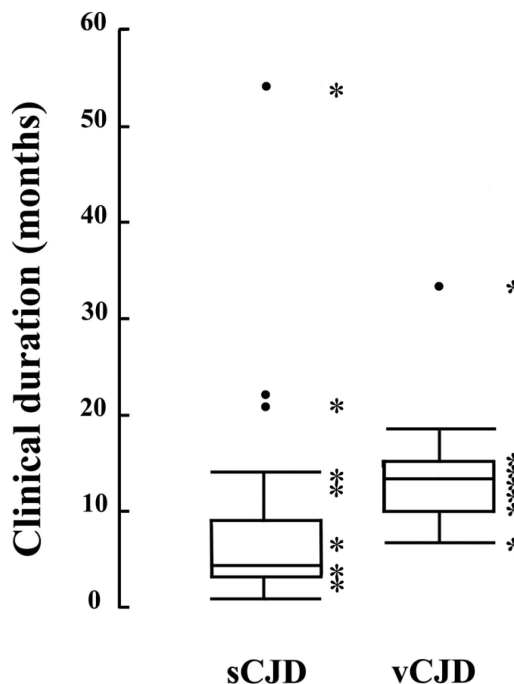


Figure 5. Clinical durations of sCJD and vCJD patients positive for PrP^{res} in muscle. The distribution of the clinical durations (in months) of the 17 vCJD patients and the 26 sCJD patients in this study are represented by box and whisker diagrams. Patients with durations of illness greater than [75th percentile + (1.5 × the interquartile range)] were defined as outliers and are indicated by filled circles. The positions of the vCJD and sCJD patients positive for PrP^{res} in muscle within these distributions are indicated with asterisks.

greater proportion of vCJD patients (8 of 17) tested positive for PrP^{res} than sCJD patients (7 of 26).

Our study was unable to confirm the suggestion of Glatzel and colleagues⁸ of a bias toward atypical *PRNP* gene codon 129 genotype and PrP^{res} isotype combinations in cases that tested positive for PrP^{res} in muscle. Two of the seven muscle PrP^{res}-positive patients had the most common PrP^{res} subtype MM1, whereas three had the rarer subtype MV2A (Table 1).^{20,22} The two growth hormone-associated iatrogenic CJD patients identified as positive for muscle PrP^{res} both had clinical durations (7 and 9 months) and codon 129/PrP^{res} isotype combinations (VV2 and MV2) that are typical for this form of the disease.

Analysis of Heart Muscle from CJD Patients for PrP^{res}

Heart muscle was available for analysis from 12 sCJD patients in our cohort, including three of the seven sCJD patients that were positive for PrP^{res} in skeletal muscle. Similarly, heart muscle from 12 vCJD patients from our cohort including four that had tested positive for skeletal muscle PrP^{res} were tested by PET blotting and Western blotting. Cardiac muscle from one iCJD patient (I5) was also tested. We obtained no positives using either technique. Our results are therefore in agreement with previous analyses of heart tissue from CJD patients by Western blotting.^{9,10}

Discussion

In their recent article Glatzel and colleagues⁸ were the first to demonstrate PrP^{res} (the proteinase K-resistant core fragment of PrP^{Sc}) in the muscle and spleen of approximately a third of sCJD patients. In this study we take these findings further by showing that 1) PrP^{res} accumulates in the skeletal muscle of acquired as well as idiopathic CJD, including vCJD and growth hormone-associated iCJD; 2) the presence of PrP^{res} in sCJD muscle is not exclusive to just the one population and is not the result of an etiology unique to Swiss sCJD patients; 3) the presence of abnormal pathogenic PrP^{Sc} in skeletal muscle from sCJD patients can be confirmed using two different techniques; 4) PrP^{res} can be localized to structures within skeletal muscle that resemble nerves; and 5) PrP^{res} is not detected in heart muscle from CJD patients using the same set of techniques. In support of these results, PrP^{res} has recently been reported (in abstract form) in skeletal muscle from an Italian patient with vCJD.²⁷ Together, these findings contrast markedly with previous studies of CJD patients by our laboratory and others, concluding that PrP^{res} was primarily undetectable in tissues outside the CNS and PNS (and additionally the lymphoid system in vCJD).^{9,10} Our ability to now detect PrP^{res} in muscle is probably due to increases in the sensitivity of both our PET blot technique²⁴ and our Western blotting method; in the latter case the main improvements were a more thorough disruption of the tissue and the introduction of the NaPTA precipitation step.

Heterogeneous Distribution of PrP^{res} in Skeletal Muscle

The results from our Western blot and PET blot analyses showed that PrP^{res} had a heterogeneous distribution within the muscle tissue from these patients, which was also observed in muscles of Swiss sCJD patients⁸ and sheep naturally infected with scrapie.²⁸ This heterogeneity makes estimating the levels of PrP^{res} in muscle difficult. However, in the positive muscle samples the signals obtained for 50 mg of muscle tissue were equivalent to 0.1 mg of vCJD brain (cerebral cortex) used as a positive control (Figures 1 to 3). This suggests that the PrP^{res} levels in positive muscle samples were ~1/500 that of vCJD cerebral cortex.

The heterogeneity and paucity of PrP^{res} within the skeletal muscle tissue of CJD patients may explain why no positive PET blots were obtained for 17 vCJD patients, even though eight of these patients were positive by Western blot. Separate samples of muscle tissue taken from the cases were analyzed using these three techniques. Clearly, the size, location and numbers of samples taken from a given specimen from a human patient will have a direct bearing on the likelihood of a positive result. This underscores the need to take multiple samples and use the most highly sensitive techniques available when assessing peripheral tissues for the presence of PrP^{res}.

Glatzel and colleagues⁸ proposed a positive correlation between the duration of disease and the detection of PrP^{res} in muscle. In our study the sCJD patients positive for muscle PrP^{res} tended to have clinical durations above the median average for this group. No such correlation was seen within our cohort of vCJD patients. However, vCJD overall is characterized by a longer clinical duration, and it is noteworthy that a greater proportion of vCJD patients were positive for PrP^{res} in muscle than sCJD patients. This suggests that a longer clinical duration may increase the likelihood of detecting PrP^{res} in muscle. However, we were unable to see a bias toward less common combinations of *PRNP* codon 129 genotype and brain PrP^{res} subtype for the patients positive for PrP^{res} in muscle.

Biochemical Profile of PrP^{res} in Muscle

A clear distinction was seen between muscle and CNS PrP^{res} in terms of the glycosylation pattern. In the positive sCJD and iCJD cases, the fastest migrating unglycosylated fragment was underrepresented in the muscle compared with PrP^{res} from the CNS. Tissue-specific effects on the glycoform ratio of PrP^{res} have been observed previously by our laboratory and others.^{9,10} Interestingly, a predominance of the di- and monoglycosylated bands is also seen in muscle PrP^{res} from the case of concurrent sCJD and inclusion body myositis reported by Kovacs and colleagues.²⁹ In the positive vCJD patients, the diglycosylated fragment of PrP^{res} did not always predominate in contrast to PrP^{res} in brain and other tissues in vCJD patients.^{9,10} This suggests that the relationship between agent strain and the PrP^{res} glycosylation pattern is not straightforward and is influenced by the tissue in which PrP^{res} has accumulated.

Localization of PrP^{res} to Musculoskeletal Nerve Fibers

With the PET blot method, muscle sections positive for PrP^{res} from the three sCJD patients and the iCJD patient all showed patterns of PrP^{res} deposition consistent with nerve fibers, with no apparent labeling of skeletal muscle fibers, motor end plates, or muscle spindles. In all four patients, this was supported by immunohistochemistry analysis of muscle sections using antibodies to label Schwann cells and axons. Although the PET blot technique may not be able to demonstrate all PrP^{res} in these tissues, it clearly indicates that the most abundant PrP^{res} deposition is in the intramuscular nerve fibers.

Cardiac muscle differs from skeletal muscle in its structure, physiology, and innervation; one or more of these factors may underlie the absence of detectable PrP^{res} in the heart samples from 25 patients with vCJD, sCJD, or iCJD examined by Western blotting or PET blotting. However, a recent report has suggested that a dilated cardiomyopathy in a patient with sCJD may have been induced by the presence of abnormal prion protein, leaving open the question of whether PrP^{Sc} is present in the heart in very rare cases of CJD.³⁰

Our study is the first to demonstrate PrP^{Sc} in musculoskeletal nerves from CJD patients. Previous analyses of PrP^{Sc} deposition in the peripheral nervous system of CJD patients gave mixed results. Primate transmission studies failed to identify prion infectivity in the peripheral nerve of sCJD patients.¹² Western blot analysis has showed PrP^{Res} in the trigeminal and dorsal root ganglia, but not peripheral or sciatic nerve in both sCJD and vCJD patients.^{9,10} However, PrP^{Res} has now been detected in the stellate and celiac ganglia of vCJD but not sCJD patients.³¹ This supports a role for gut-associated sympathetic neurons in the propagation and centripetal spread of vCJD prions in humans after oral contamination. Most recently Ishida and colleagues³² have reported the immunohistochemical detection of PrP^{Sc} deposits in the peripheral nerves of dural graft-associated iCJD patients, suggesting centrifugal spread of prions from the CNS to the periphery. However, a centrifugal spread may not be relevant in vCJD or iCJD cases after growth hormone therapy because a peripheral route of primary infection is likely to apply in both groups. It remains to be seen whether the invasion of muscle tissue by PrP^{Sc} occurs at an early stage in the progression of the disease or indeed whether it occurs in presymptomatic individuals. In this context, it should be noted that no PrP^{Res} was detected by NaPTA precipitation/Western blot in the muscle tissue taken at autopsy from a patient with preclinical vCJD infection in the spleen as reported previously.¹⁶

Comparison with Animal Prion Disease Models

Muscle PrP^{Res} was more readily detected in mouse³³ and hamster³⁴ prion disease models compared with our study of human CJD patients. In contrast to studies of muscle from sheep intracerebrally challenged with scrapie,²⁸ we could not detect an association of PrP^{Res} with muscle spindles. Furthermore, PrP^{Res} was not found to be associated with muscle fibers in human CJD patients in contrast to muscles from hamsters orally infected with 263K scrapie.³⁴ The greater and more widespread deposition of PrP^{Res} in the scrapie hamster and scrapie sheep models may be a consequence of increased infection pressure and/or infectivity titer in these disease models compared with human prion diseases. The infectivity titers of ~10⁸ to 10⁹ ID₅₀/g determined for brain in the mouse³³ and hamster³⁵ scrapie models are much greater than the estimate of 10⁵ ID₅₀/g for CJD brain,¹² even when we take into account the inevitable species barrier when human CJD tissues are bioassayed. For these reasons experimental ovine and rodent disease models may exaggerate the extent of PrP^{Res} in the peripheral tissue when compared with natural disease states. Our findings caution against an overreliance on these models when assessing human disease.

Public Health Implications

There is a strong correlation between the presence of PrP^{Sc} and infectivity in prion diseases. Hence these studies would suggest that prion infectivity is indeed present

in the skeletal muscle tissue from all forms of CJD, both acquired and sporadic, albeit at levels two orders of magnitude lower than those found in the CNS. The presence of PrP^{Sc} in skeletal muscle suggests that the potential risk of iatrogenic spread may be a cause for concern for a range of surgical procedures, although further investigation is required to allow a full risk assessment.

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