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Detection and Localization of PrPSc 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 PrPSc 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 PrPSc in skeletal muscle from a quarter of Swiss patients with sporadic CJD (sCJD). This posed the question of whether PrPSc 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 PrPSc in muscle tissue specimens taken at autopsy from 49 CJD patients in the United Kingdom. These techniques identified muscle PrPSc in 8 of 17 vCJD, 7 of 26 sCJD, and 2 of 5 iCJD patients. Paraffin-embedded tissue blotting analysis showed PrPSc in skeletal muscle in localized anatomical structures that had the morphological and immunohistochemical characteristics of nerve fibers. The detection of PrPSc 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 pathogenesis of prion diseases is the conversion of the cellular prion protein PrPC to the abnormal disease-associated form PrPSc. According to the prion hypothesis, PrPSc is the principle or sole component of the infectious agent, and the accumulation of PrPSc 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 PrPC to PrPSc 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.

PrPSc differs from PrPC in its relative resistance to proteases and is often referred to as PrPres after partial proteolytic degradation. Although PrPres accumulation occurs primarily in the brain, PrPres has also been detected in the peripheral tissues of CJD patients. 8–10 This is particularly the case for vCJD in which PrPres 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\textsuperscript{res} accumulation (and by implication infectivity) was thought to be primarily confined to the CNS.\textsuperscript{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.\textsuperscript{12} Also, there was always the possibility that the detection of low levels of PrP\textsuperscript{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 colleagues\textsuperscript{8} who were able to detect PrP\textsuperscript{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\textsuperscript{res} with sodium phosphotungstic acid (NaPTA).

The detection of PrP\textsuperscript{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.\textsuperscript{13} The study by Glatzel and colleagues\textsuperscript{8} requires confirmation in sCJD patients from other populations. The study was purely biochemical in nature and did not investigate the localization of PrP\textsuperscript{res} within the muscle tissue. Lastly, the work of Glatzel and colleagues\textsuperscript{8} raises the question of whether PrP\textsuperscript{res} is present in the muscle of patients with other forms of CJD, most notably vCJD, where the extent of peripheral PrP\textsuperscript{res} tends to be greater.\textsuperscript{9,10} Because of the oral route of infection in vCJD and the presumed long incubation period,\textsuperscript{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.\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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.\textsuperscript{17,18} The protease-resistant prion protein (PrP\textsuperscript{res}) isotype found in brain was classified as type 1, 2A, or 2B as previously described according to the nomenclature of Parchi and colleagues.\textsuperscript{19–22} The polymorphic status of codon 129 of the prion protein gene PRNP of each case was determined by restriction fragment length polymorphism.\textsuperscript{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 colleagues\textsuperscript{8} and Wadsworth and colleagues\textsuperscript{10} previously, with a few modifications. Briefly, 10% w/v extracts were made of 65- to 100-kg 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 (Qiogene, Cambridge, UK). The samples were then cleared by centrifugation at 5200 \( \times g \) 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 MgCl\(_2\) 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 \( \mu l\) of a stock solution of 4% w/v NaPTA and 170 mmol/L MgCl\(_2\), 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 \( \times g \) for 30 minutes at 37°C. The resultant supernatant was discarded and the pellets were resuspended in 20 \( \mu l\) of 0.1% w/v sarkosyl in PBS, pH 7.4, and digested with 50 \( \mu g/ml\) 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 to antimouse IgG was used as the secondary antibody. Immunoreactive bands were visualized using horseradish peroxidase substrate (Vector Laboratories, Peterborough, UK). The specificity of all Western blotting results was confirmed by the use of a recombinant inactive prion protein (rPrP) control (KPL, Brussels, Belgium).
peroxidase-conjugated anti-mouse IgG F(ab')\textsubscript{2} fragment (Amer sham Biosciences, Amersham, UK) was used at a dilution of 1 in 40,000 for 1 hour. The detection reagent used was SuperSignal West Femto 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\textsuperscript{res} was estimated by reference to IgG-binding MagicMark XP Western protein standards (Invi trogen), and standard PrP\textsuperscript{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.\textsuperscript{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 \mu 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\textsuperscript{res} that can lead to an underrepresentation of one of the three characteristic bands observed on the Western blots.\textsuperscript{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\textsuperscript{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\textsuperscript{res} in paraffin sections of cardiac and skeletal muscles was investigated using the highly sensitive PET blot method.\textsuperscript{24,25} To establish the cellular location of PrP\textsuperscript{res} deposits, conventional immunohistochemistry was performed to compare the localization of a number of cellular markers with the distribution of PrP\textsuperscript{res}. Serial 5-\mu 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-\mu 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 previously\textsuperscript{24} using a modified version of the method of Schulz-Schaeffer and colleagues\textsuperscript{25} Briefly, sections mounted on nitrocellulose were dewaxed before an overnight digestion in 25 \mu 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\textsuperscript{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 microwaveing 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\textsuperscript{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\textsuperscript{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\textsuperscript{res} (Table 1), suggesting that PrP\textsuperscript{res} has a heterogeneous distribution within the muscle tissue. The samples that were positive contained PrP\textsuperscript{res} at levels approximately equal to the positive control, which contained 100 \mu g of vCJD brain homogenate. Because each sample is the NaPTA

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**PrP\textsuperscript{res} in CJD Muscle**

**AJP March 2006, Vol. 168, No. 3**
precipitate from 50 mg of wet weight of tissue, we estimate that the levels of PrPres in these positive muscle samples were \( \frac{1}{500} \) that of vCJD brain.

Patients with sCJD

Using this same Western blotting technique, 6 of 26 patients with definite sCJD were identified as being positive for skeletal muscle PrPres (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 PrPres in all three samples taken. Interestingly, the unglycosylated PrPres fragment was under-represented in the muscle from patient S10 when compared with PrPres from the CNS (spinal cord) of this patient (Figure 2). The bands identified as being PrPres 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 PrPres by Western Blotting (WB) and PET Blotting

<table>
<thead>
<tr>
<th>Case</th>
<th>WB (no. positive for PrPres/total)</th>
<th>PET (no. positive for PrPres/total)</th>
<th>Codon 129</th>
<th>Brain PrPres type</th>
</tr>
</thead>
<tbody>
<tr>
<td>vCJD patients (17 analyzed)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V2</td>
<td>(1/4)</td>
<td>(0/1)</td>
<td>MM</td>
<td>2B</td>
</tr>
<tr>
<td>V5</td>
<td>(3/7)</td>
<td>(0/1)</td>
<td>MM</td>
<td>2B</td>
</tr>
<tr>
<td>V6</td>
<td>(1/2)</td>
<td>(0/1)</td>
<td>MM</td>
<td>2B</td>
</tr>
<tr>
<td>V8</td>
<td>(1/7)</td>
<td>(0/1)</td>
<td>MM</td>
<td>2B</td>
</tr>
<tr>
<td>V9</td>
<td>(2/4)</td>
<td>(0/1)</td>
<td>MM</td>
<td>2B</td>
</tr>
<tr>
<td>V12</td>
<td>(1/13)</td>
<td>(0/1)</td>
<td>MM</td>
<td>2B</td>
</tr>
<tr>
<td>V13</td>
<td>(1/3)</td>
<td>(0/1)</td>
<td>MM</td>
<td>2B</td>
</tr>
<tr>
<td>sCJD patients (26 analyzed)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S7</td>
<td>(1/2)</td>
<td>(1/1)</td>
<td>MV</td>
<td>2A (+1)</td>
</tr>
<tr>
<td>S8</td>
<td>(1/2)</td>
<td>(0/1)</td>
<td>MM</td>
<td>1 + 2A</td>
</tr>
<tr>
<td>S10</td>
<td>(3/3)</td>
<td>(1/1)</td>
<td>MV</td>
<td>1</td>
</tr>
<tr>
<td>S11</td>
<td>(2/7)</td>
<td>(0/1)</td>
<td>MM</td>
<td>1</td>
</tr>
<tr>
<td>S14</td>
<td>(0/3)</td>
<td>(1/1)</td>
<td>MV</td>
<td>2A</td>
</tr>
<tr>
<td>S24</td>
<td>(1/2)</td>
<td>(0/1)</td>
<td>MM</td>
<td>1</td>
</tr>
<tr>
<td>S25</td>
<td>(1/2)</td>
<td>(0/1)</td>
<td>MV</td>
<td>2A</td>
</tr>
<tr>
<td>iCJD patients (5 analyzed)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>I4</td>
<td>(4/5)</td>
<td>(0/1)</td>
<td>VV</td>
<td>2A</td>
</tr>
<tr>
<td>I5</td>
<td>(0/3)</td>
<td>(1/1)</td>
<td>MV</td>
<td>2A</td>
</tr>
</tbody>
</table>

The number of samples tested (no. positive for PrPres/total) is indicated in parentheses. The positive scores are indicated in bold type.
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 PrPres 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 PrPres in the spiked positive control, which was the NaPTA precipitate from 100 μg of vCJD (PrPres type MM2B) brain extract diluted in 50 mg of non-CJD control muscle homogenate (Figure 3). As shown in Figure 3, PrPres obtained after NaPTA precipitation and PK digestion in the spiked positive control (vCJD, spike) consistently had a higher apparent molecular weight than PrPres from vCJD extract prepared and PK-digested without prior NaPTA precipitation (vCJD, +). As shown previously NaPTA causes this effect when applied to PrPSc before proteolysis by altering the PK cleavage site, resulting in the generation of larger PrPres 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 PrPres in the muscle tissue.

Figure 3. Western blots of iCJD patient muscle samples positive for PrPres. Three samples of muscle from an iCJD patient (I4) are compared with 100 μg 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 μg and 200 μg, 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 previously there is a slight upward shift in the mobility of PrPres after NaPTA precipitation (+vCJD spike lane).

Localization of PrPres in Muscle Tissue

PET Blot Analysis of CJD Patients

To investigate the muscle tissue heterogeneity we have used PET blotting to localize PrPSc 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, S10...
and S14) were found to be positive for PrPres (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 PrPres to seven (of 26). PET blotting also showed PrPres 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, PrPres 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 PrPres 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 PrPres 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 PrPres-Positive Skeletal Muscle**

The pathological, genetic, and clinical data for the patients positive for muscle PrPres were analyzed against the data for all of the CJD patients in this study. The data on the PRNP codon 129 genotype and brain PrPres iso-type combinations and duration of illness of the vCJD, sCJD, and iCJD patients identified as positive for PrPres 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. 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 PrPres 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 months), and a greater proportion of vCJD patients (8 of 17) tested positive for PrPres than sCJD patients (7 of 26). Our study was unable to confirm the suggestion of Glatzel and colleagues of a bias toward atypical PRNP gene codon 129 genotype and PrPres isotype combinations in cases that tested positive for PrPres in muscle. Two of the seven muscle PrPres-positive patients had the most common PrPres subtype MM1, whereas three had the rarer subtype MV2A (Table 1). The two growth hormone-associated iatrogenic CJD patients identified as positive for muscle PrPres both had clinical durations (7 and 9 months) and codon 129/PrPres isotype combinations (VV2 and MV2) that are typical for this form of the disease.

**Analysis of Heart Muscle from CJD Patients for PrPres**

Heart muscle was available for analysis from 12 sCJD patients in our cohort, including three of the seven sCJD patients that were positive for PrPres in skeletal muscle. Similarly, heart muscle from 12 vCJD patients from our cohort including four that had tested positive for skeletal muscle PrPres 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 colleagues\(^8\) were the first to demonstrate PrPres (the proteinase K-resistant core fragment of PrP\(^\text{Sc}\)) in the muscle and spleen of approximately a third of sCJD patients. In this study we take these findings further by showing that 1) PrPres accumulates in the skeletal muscle of acquired as well as idiopathic CJD, including vCJD and growth hormone-associated iCJD; 2) the presence of PrPres 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\(^\text{Sc}\) in skeletal muscle from sCJD patients can be confirmed using two different techniques; 4) PrPres can be localized to structures within skeletal muscle that resemble nerves; and 5) PrPres is not detected in heart muscle from CJD patients using the same set of techniques. In support of these results, PrPres 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 PrPres was primarily undetectable in tissues outside the CNS and PNS (and additionally the lymphoid system in vCJD).\(^9\) Our ability to now detect PrPres in muscle is probably due to increases in the sensitivity of both our PET blot technique\(^24\) 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 PrPres in Skeletal Muscle

The results from our Western blot and PET blot analyses showed that PrPres had a heterogeneous distribution within the muscle tissue from these patients, which was also observed in muscles of Swiss sCJD patients\(^8\) and sheep naturally infected with scrapie.\(^28\) This heterogeneity makes estimating the levels of PrPres 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 PrPres levels in positive muscle samples were ~1/500 that of vCJD cerebral cortex.

The heterogeneity and paucity of PrPres 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 PrPres.

Glatzel and colleagues\(^8\) proposed a positive correlation between the duration of disease and the detection of PrPres in muscle. In our study the sCJD patients positive for muscle PrPres 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 PrPres in muscle than sCJD patients. This suggests that a longer clinical duration may increase the likelihood of detecting PrPres in muscle. However, we were unable to see a bias toward less common combinations of PRNP codon 129 genotype and brain PrPres subtype for the patients positive for PrPres in muscle.

Biochemical Profile of PrPres in Muscle

A clear distinction was seen between muscle and CNS PrPres 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 PrPres from the CNS. Tissue-specific effects on the glycoform ratio of PrPres have been observed previously by our laboratory and others.\(^9\) Interestingly, a predominance of the di- and monoglycosylated bands is also seen in muscle PrPres 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 PrPres did not always predominate in contrast to PrPres in brain and other tissues in vCJD patients.\(^9\) This suggests that the relationship between agent strain and the PrPres glycosylation pattern is not straightforward and is influenced by the tissue in which PrPres has accumulated.

Localization of PrPres to Musculoskeletal Nerve Fibers

With the PET blot method, muscle sections positive for PrPres from the three sCJD patients and the iCJD patient all showed patterns of PrPres 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 PrPres in these tissues, it clearly indicates that the most abundant PrPres 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 PrPres 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 PrPSc is present in the heart in very rare cases of CJD.\(^30\)
Our study is the first to demonstrate PrPSc in musculoskeletal nerves from CJD patients. Previous analyses of PrPSc 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. Western blot analysis has showed PrPres in the trigeminal and dorsal root ganglia, but not peripheral or sciatic nerve in both sCJD and vCJD patients. However, PrPres has now been detected in the stellate and celiac ganglia of vCJD but not sCJD patients. This supports a role for gut-associated sympathetic neurons in the propagation and centrifugal spread of vCJD prions in humans after oral contamination. Most recently Ishida and colleagues have reported the immunohistochemical detection of PrPSc 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 PrPSc 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 PrPres 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.

Comparison with Animal Prion Disease Models

Muscle PrPres was more readily detected in mouse and hamster prion disease models compared with our study of human CJD patients. In contrast to studies of muscle from sheep intracerebrally challenged with scrapie, we could not detect an association of PrPres with muscle spindles. Furthermore, PrPres was not found to be associated with muscle fibers in human CJD patients in contrast to muscles from hamsters orally infected with 263K scrapie. The greater and more widespread deposition of PrPres 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 \(10^4\) to \(10^9\) ID50/g determined for brain in the mouse and hamster scrapie models are much greater than the estimate of \(10^5\) to \(10^6\) ID50/g for CJD brain, 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 PrPres 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 PrPSc 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 PrPSc 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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