Neurobiology

Creutzfeldt-Jakob Disease (CJD) with a Mutation at Codon 148 of Prion Protein Gene

Relationship with Sporadic CJD

Manuela Pastore,* Steven S. Chin,[†]
Karen L. Bell,[‡] Zhiqian Dong,*[§] Qiwei Yang,*
Lizhu Yang,*[§] Jue Yuan,*[§] Shu G. Chen,*[§]
Pierluigi Gambetti,*[§] and Wen-Quan Zou*[§]

From the Department of Pathology, Case Western Reserve University* and National Prion Disease Pathology Surveillance Center,[§] Cleveland, Ohio; the University of Utah Health Science Center,[†] Salt Lake City, Utah; and Columbia University College of Physicians & Surgeons,[‡] New York, New York

Creutzfeldt-Jakob disease (CJD), the most common human prion disease, includes sporadic (s) and familial (f) forms. Regardless of etiology, both forms are thought to share the pathogenic mechanism whereby the cellular prion protein (PrP^C) converts into its pathogenic isoform (PrP^{Sc}). While PrP^C conversion is thought to be random in sCJD, conversion in fCJD is facilitated by the congenital presence of mutated PrP. Differences in PrP genotype (*PRNP*) and in conversion circumstances lead to PrP^{Sc} with distinct characteristics that elicit different disease phenotypes. Here, we describe a case of fCJD with a substitution of histidine (H) for arginine (R) at codon 148 (R148H) and heterozygosity of the methionine/valine (M/V) polymorphic codon 129, with the 129M allele coupled with the mutation. The disease phenotype and all major characteristics of PrP^{Sc} of fCJD^{R148H} were virtually indistinguishable from those of sCJDMV2, which has features different from those of any other sCJD. Therefore, despite the differences in etiology, *PRNP*, and conversion process, the two forms of PrP^{Sc} had similar characteristics. Furthermore, comparison of fCJD^{R148H} with a recently reported case carrying R148H and homozygosity at codon 129 suggests that codon 129 coupled with the mutation as well as that located on the normal allele can modify major phenotypic and PrP^{Sc} features of fCJD^{R148H}. (Am J Pathol 2005, 167:1729–1738)

Human prion diseases are composed of sporadic and familial forms as well as forms caused by infection.^{1,2} Five types of Creutzfeldt-Jakob disease (CJD) and fatal insomnia are the sporadic forms that can be distinguished based on the characteristics of the disease phenotype.³ Fifty-six mutations, including the recently reported substitution of histidine (H) for arginine (R) at codon 148 (R148H) of the prion protein gene (*PRNP*), are known to be associated with familial prion diseases.^{4–6} Furthermore, it has been shown that the common methionine/valine (M/V) polymorphism at codon 129 of *PRNP* may modify the features of the disease phenotype associated with a *PRNP* mutation, depending on which of the two polymorphic codons is coupled with the mutation.^{4,7} Of a total of 62 disease-associated *PRNP* haplotypes reported, 15 are associated with a phenotype that belongs to the group of CJD.^{4–6} Despite the high number of pathogenic haplotypes, the distinct phenotypes associated with the familial CJD (fCJD) are, surprisingly, relatively limited in number, and they often appear to mimic the phenotypes of sporadic CJD (sCJD).^{8,9} This observation raises the important issue related to the pathogenesis of prion diseases as to how the disease phenotype is related to the molecular characteristics of the pathological prion protein (PrP^{Sc}), the only known component in the infectious pathogen.

All prion diseases are thought to share the same pathogenic mechanism that involves a conformational transition of α -helix into β -sheet structure in prion protein (PrP).¹⁰ The normal or cellular PrP (PrP^C) interacts with the pathological and infectious PrP^{Sc} and thus adopts the same conformation as the PrP^{Sc}.¹⁰ When a sufficient

Supported by the National Institutes of Health grants AG-14359 and AG08702, by the Center for Disease Control and Prevention contract UR8/CCU515004, and by the Britton Fund.

Accepted for publication August 25, 2005.

A portion of this study has been previously reported as an abstract.⁵

Address reprint requests to Pierluigi Gambetti, M.D., Department of Pathology, Case Western Reserve University, 2085 Adelbert Road, Cleveland, OH 44106. E-mail: pxg13@case.edu.

amount of PrP^{Sc} is generated, the disease becomes symptomatic. In sporadic prion diseases, PrP^{Sc} is thought to form spontaneously and randomly due to a co- or posttranslational error in PrP processing. In familial prion diseases, the etiology is thought to be different; these diseases are believed to result from the conversion of the mutant PrP (PrP^M), a molecule that is unstable because of the presence of the mutation.⁴ The PrP^C to PrP^{Sc} conversion results in the formation of the PrP^{Sc} species with different physicochemical characteristics probably reflecting distinct conformations also identified as "prion strains."^{11–14} In familial prion diseases, distinct prion strains are seemingly determined by the PrP genotype and are expected to be associated with different disease phenotypes.¹

We have observed a family carrying a PrP mutation replacing arginine with histidine at position 148 of PrP (R148H), a region that, until recently, had remained invariant. The disease phenotype of the proband has not been previously observed in fCJD but mimics a distinct phenotype associated with a type of sCJD characterized by the presence of heterozygosity at codon 129 and the presence of the PrP^{Sc} type 2 (sCJDMV2).³ This observation together with another case of fCJD associated with the R148H mutation (fCJD^{R148H}) that was reported soon after the completion of our study⁶ offers the opportunity to gain insight into the relationship among genotypes, PrP^{Sc} molecular characteristics, and disease phenotypes. We performed a comparative study of the phenotype and of the characteristics of PrP^{Sc} present in our case of fCJD^{R148H} and those associated with sCJDMV2. We show that both phenotypic and PrP^{Sc} characteristics are indistinguishable in fCJD^{R148H} and sCJDMV2 despite their allegedly different etiology.

Materials and Methods

Reagents and Antibodies

Proteinase K (PK), phenylmethylsulfonyl fluoride (PMSF), and guanidine hydrochloride (GdnHCl) were purchased from Sigma Chemical Co. (St. Louis, MO). Peptide N-glycosidase F (PNGase F) was purchased from New England Biolabs Inc. (Beverly, MA). Urea, CHAPS, DL-dithiothreitol (DTT), iodoacetamide, tributylphosphine (TBP), Ampholine pH 3–10, and immobilized pH gradient (IPG) strips (pH 3 to 10, 11 cm long) were from Bio-Rad (Richmond, CA). Reagents for enhanced chemiluminescence (ECL Plus) were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Anti-PrP antibodies, including rabbit antisera to human PrP residues 23 to 40 (anti-N) and residues 220 to 231 (anti-C),^{15,16} as well as mouse monoclonal antibody 3F4 against human PrP residues 109 to 112¹⁷ were used. All other chemicals were purchased from Sigma unless specified otherwise.

Clinical History and Brain Tissue Specimens

Clinical history was collected and examined. Consent to use autopsy material for research purposes was obtained

for all samples. Autopsy of the proband was performed within 20 hours from death. Cases of proven sCJDM1 ($n = 3$) and sCJDMV2 ($n = 3$), diagnosed at the National Prion Disease Pathology Surveillance Center, were used as controls.

Brain was removed at autopsy and one-half was immediately frozen and stored at -80°C . The frozen and fixed brain tissues were sent to the National Prion Disease Pathology Surveillance Center for diagnosis of the disease. Frozen tissues from frontal cortex (FC), temporal cortex (TC), parietal cortex (PC), occipital cortex (OC), neocortices, hippocampus (HI) (CA 1 region), entorhinal cortex (EC), neostriatum (ST) (nucleus caudatus), thalamus (TH) (mediodorsal nucleus), substantia nigra (SN), midbrain periventricular gray (PG), locus ceruleus (LC), medulla (ME) (olive), and cerebellum (hemisphere) (CE) were determined by Western blotting for PrP. The remaining tissue was fixed in formalin for 10 days and kept in 98% formic acid for 1 hour and again in formalin until sampling for neuropathological examination and PrP immunohistochemistry. The paraffin blocks of tissues from FC, TC, PC, OC, neocortices, HI (CA 1 region), EC, basal ganglia (anterior and posterior) (BG), TH (anterior and posterior), pons (PN), and CE (hemisphere) were prepared for histology and immunohistochemistry.

Molecular Genetic Analysis

Genomic DNA was extracted from frozen brain tissue or blood specimens. Blood specimens for genetic analysis were obtained from the probanda and her family. The M/V polymorphism at codon 129 and mutation of *PRNP* coding region were determined as described previously.^{7,18,19}

PrP Immunohistochemistry

The sections were deparaffinized, rehydrated, and immersed in 98% formic acid for 1 hour at room temperature.^{3,19} Endogenous peroxidase was blocked by immersion in 8% hydrogen peroxide in methanol for 10 minutes. Sections were completely immersed in 1.5 mmol/L hydrochloric acid and microwaved for 10 minutes. After rinsing, they were incubated with 3F4 at 1:600, washed, and incubated with secondary antibody (goat anti-mouse, Cappel, 1:50) followed by incubation with mouse PAP complex (Sternberger; Meyer Immunocytochemicals Inc., Jarrettsville, MD; 1:250). Diaminobenzidine tetrahydrochloride was used to visualize the immunoreactivity.

Histopathological Examinations

Semiquantitative evaluation of the prion-related brain lesions 1) spongiform degeneration, 2) neuronal loss, and 3) gliosis was performed on hematoxylin and eosin-stained sections as described previously.^{3,19} Spongiform degeneration was scored on a 0 to 4 scale (not detectable, mild, moderate, severe, and confluent); astrogliosis and neuronal loss were scored individually on a 0 to 3 scale (not detectable, mild, mod-

erate, and severe). Ten brain regions from each case were examined. The brains from three cases of sCJDMV2 and three cases of sCJDMM1 were used as references. The scores of each of the lesions obtained at each of the 10 brain regions were added, values from each of the three sCJDMV2 and sCJDMM1 reference cases were averaged, SD was determined at each region, and data were plotted as a function of the brain regions and compared with the values obtained from the proband.^{3,19}

Preparation of Brain Homogenate and P2 Fraction

Brain homogenate was prepared in 9 volumes of lysis buffer (10 mmol/L Tris, 150 mmol/L NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, and 5 mmol/L EDTA, pH 7.5) in the presence of 1 mmol/L PMSF and stored in aliquots at -80°C . When required, brain homogenate was centrifuged at $1000 \times g$ for 10 minutes at 4°C . The detergent-insoluble fraction (P2) was prepared as described previously.²⁰ In brief, supernatant (S1) from $1000 \times g$ centrifugation was subjected to further ultracentrifugation at $100,000 \times g$ for 1 hour at 4°C . The new supernatant (S2, detergent-soluble fraction) was recovered and stored at -80°C . The pellet (P2) was resuspended in lysis buffer.

Western Blot Analysis

Quantitative Western blot analysis was performed as previously described.²¹ Briefly, total brain homogenates were digested with PK (100 $\mu\text{g}/\text{ml}$) for 1 hour at 37°C . Digestion was terminated by the addition of PMSF (3 mmol/L final concentration) and boiling for 10 minutes in electrophoresis sample buffer (3% SDS, 2 mmol/L EDTA, 10% glycerol, and 2.5% β -mercaptoethanol in 62.5 mmol/L Tris, pH 6.8). Samples treated with or without PK digestion were resolved on 15% Tris-HCl precast gel (Bio-Rad), and proteins were transferred to Immobilon-P membrane (PVDF; Millipore) for 2 hours at 70 V.

For probing PrP, the membranes were incubated for 2 hours at room temperature with 3F4 (1:40,000), anti-C (1:4000), or anti-N (1:4000) antibody. The PrP bands or PrP spots were visualized on Kodak film by the ECL Plus as described by the manufacturer. Intensities of the signal generated by the ECL Plus were determined with the Epi Chemi II Darkroom (UVP Inc., Upland, CA) and analyzed with the Lab Works 4.0 Image Acquisition and Analysis Software (UVP Inc.) with appropriate correction for background absorption.

Two-Dimensional Gel Electrophoresis

Two-dimensional (2D) gel electrophoresis was performed as described by the supplier using the PROTEIN IEF cell (Bio-Rad) with minor modifications.²² For the first dimension, P2 fractions boiled in the electrophoresis sample buffer were precipitated by fivefold volume of prechilled methanol at -20°C for 2 hours,

followed by centrifugation at $16,000 \times g$ for 20 minutes at 4°C . Pellets were resuspended in reducing buffer (8 mol/L urea, 2% CHAPS, 5 mmol/L tributylphosphine, and 20 mmol/L Tris, pH 8.0) for 1 hour at room temperature and then incubated with 20 mmol/L iodoacetamide for 1 hour. After incubation with methanol and subsequent centrifugation, the pellets were resuspended in 200 μl of rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 1% DTT, 1% CHAPS, 1% Triton X-100, 1% Ampholine, pH 3 to 10, and trace amounts of bromophenol blue). The samples dissolved in rehydration buffer were incubated with the IPG strips for 14 hours at room temperature with shaking. The rehydrated strips were focused for about 40 kVh using the PROTEIN IEF cell. For the second dimension, the focused IPG strips were equilibrated for 15 minutes in equilibration buffer 1 (6 mol/L urea, 2% SDS, 20% glycerol, 130 mmol/L DTT, and 375 mmol/L Tris, pH 8.8) and then in equilibration buffer 2 (6 mol/L urea, 2% SDS, 20% glycerol, 135 mmol/L iodoacetamide, and 375 mmol/L Tris, pH 8.8) for another 15 minutes. The equilibrated strips were loaded onto 8 to 16% Tris-HCl precast gel (Bio-Rad). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed as described above.

Conformational Stability Assay (CSA) of PrP^{Sc}

Aliquots of homogenates from FC were incubated with various concentrations of GdnHCl from 0 to 3.0 mol/L.^{13,23} GdnHCl stock solutions were prepared from an 8 mol/L solution diluted in water. After incubation for 1 hour at room temperature, all samples were precipitated in prechilled methanol at -20°C for 2 hours and centrifuged at $16,000 \times g$ for 20 minutes at 4°C . Pellets were resuspended in lysis buffer and treated with 100 $\mu\text{g}/\text{ml}$ PK for 1 hour at 37°C . The reaction was stopped by adding 3 mmol/L PMSF and electrophoresis sample buffer and boiled for 10 minutes. The samples were used for Western blot analysis as described above.

Mass Spectrometric Analysis of Purified PK-Resistant PrP Fragments

Brain tissue from the affected subject was used for the purification of PrP^{Sc} and generation of its PK-resistant C-terminal core fragment (PrP27–30), as described previously.^{20,24} PrP27–30 was denatured and treated with PNGase F followed by digestion with endoproteinase Asp-N.^{18,24} An aliquot of the Asp-N digest was subjected to matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) using a Bruker Biflex III mass spectrometer operated in the reflectron mode. Monoisotopic peaks of detected peptide ions were assigned automatically by the SNAP function of the Xmass software (Bruker).

Figure 1. Histopathology and immunohistochemistry. **A:** Laminar spongiform degeneration in layers V and VI of the neocortex; H&E, $\times 40$. **B** and **C:** Kuru plaques (**arrows**) in the granular cell layer of the cerebellum and in the white matter (H&E, $\times 400$). **D:** Plaque-like formations and staining of processes (**two arrows**) in the thalamus (mAb 3F4, $\times 200$). **E:** Plaque-like formations and staining along processes (**arrow**) in the cerebellar granule cell layer (mAb 3F4, $\times 100$). **F:** Higher magnification of plaque-like formations of **E** (mAb 3F4, $\times 400$). **G:** Set of plaque-like formations in the white matter of the cerebellum (**arrows**) (mAb 3F4, $\times 400$).

Results

Clinical Findings

The proband, a 62 year-old Caucasian female, began complaining of difficulty in hearing and driving in July 1998. From November 1998 to June 1999, she complained of increasing fatigue and sleepiness. Her family reported balance and gait impairments associated with dizziness and mental disturbances that affected activities of her daily living. Her memory also deteriorated, and she developed delusions and hallucinations.

The first neurological examination in June 1999 revealed markedly impaired gait with postural instability and mild dysmetria in finger/nose/finger maneuver but no myoclonus. She was cognitively impaired with a Modified Mini Mental test score of 38 out of 57. She was oriented to person and place but not to time. Her electroencephalogram and the CSF 14-3-3 protein level were normal. The patient continued to deteriorate and expired 18 months after the onset of symptoms without ever showing myoclonus or electroencephalogram periodic sharp wave complexes (PSW) seen in classical CJD. The proband's mother died at 93, from a stroke and her father from a myeloproliferative disorder at the age of 65. The proband had two sisters (70 and 66 years old), a daughter (42 years old), and a son (40 years old), all apparently in good health at the time of this study.

Histopathology and Immunohistochemistry

The neocortex showed spongiform degeneration that preferentially affected the fourth and fifth layers and, to a lesser extent, the second layer (Figure 1A). Overall, the spongiform degeneration appeared to be more severe in the frontal and parietal than temporal and occipital lobes.

The hippocampal formation presented minimal fine spongiform degeneration in the Sommer's sector and coarse spongiform degeneration at the junction between subiculum and entorhinal cortex. In the basal ganglia, the spongiform degeneration was more prominent than in the neocortex especially in the putamen, where it was associated with astrogliosis and occasional Rosenthal fibers. The thalamus was moderately affected. The cerebellum showed minimal spongiform degeneration in the molecular layer and in the dentate nucleus. The defining histological feature was the presence of kuru plaques in the granular and Purkinje cell layers of the cerebellum (Figure 1B) and in the superficial white matter (Figure 1C). Torpedoes were also present. The brain stem and the pons showed neuronal loss, gliosis, and spongiform degeneration in the tegmentum and pontine nuclei.

The PrP immunostaining was characterized by the intense staining of the kuru plaques in the granular and Purkinje cell layers of the cerebellum (Figure 1, E and F) and in the white matter (Figure 1G), by the presence of plaque-like formations often associated with the "synaptic" pattern (Figure 1D), as well as by staining around neuronal cell bodies and processes. The intensity of the immunostaining roughly matched the severity of the histological lesions. The pons showed fine synaptic staining of pontine nuclei and more intense granular staining with numerous plaque-like deposits in the tegmental region.

The Mutation in the PrP Open Reading Frame

Sequencing of the proband's *PRNP* open reading frame revealed a transition from a guanine to adenine at codon 148, resulting in the R148H mutation of PrP. In addition, the proband was heterozygous (M/V) at the polymorphic codon 129, with codon 129M coupled to the mutant codon forming the R148H-129M haplotype. Two sisters carried the R148H mutation, but they were M/M homozygous at codon 129. Both children were free of *PRNP* mutations and homozygous V/V at codon 129 (Figure 2).

Allelic Composition of PrP^{Sc} from fCJD^{R148H}

The finding that the proband was heterozygous for the R148H mutation and the M/V polymorphism at codon 129 raised the issue as to whether PrP^{Sc} only originates from the mutant allele or from both. To resolve this issue, we performed MALDI-mass spectrometric (MS) analysis of purified PrP27–30 from fCJD^{R148H} after deglycosylation with PNGase F and digestion with endoprotease Asp-N that generated the 147 to 166 prion peptide. The MALDI-MS spectrum of the treated PrP27–30 demonstrated two peptide ions with well-resolved isotopic envelopes. The monoisotopic peaks were recovered at *m/z* values of 2731.6 and 2750.5, respectively (Figure 3A). These values matched the theoretical *m/z* values of 2732.2 for mutant peptide and of 2751.3 for the wild-type peptide, indicating that both PrP^M and wild-type PrP (PrP^{Wt}) were converted into PrP^{Sc} in fCJD^{R148H} (Figure 3B).

Figure 2. Pedigree of the propoitus family.

Comparison of Pathological Phenotypes and PrP^{Sc} Features in fCJD^{R148H} and sCJD MV2

Lesion Profile

At regular microscopic examination, the histopathological and immunohistochemical features of fCJD^{R148H} looked indistinguishable from those of sCJDMV2, the only sCJD subtype with kuru plaques. This finding was con-

A

B

Asp-N peptide	Amino acid sequence	[M+H] ⁺ th	[M+H] ⁺ ex
Wild-type	DRYYRENHRYPNQVYYRPM	2751.3	2750.5
Mutant	DHYYRENHRYPNQVYYRPM	2732.2	2731.6

Figure 3. MALDI-MS data showing the allelic composition of purified PrP^{Sc} from the brain affected with fCJD^{R148H}. **A:** MALDI-MS spectrum of peptide ions (m/z range, 2720 to 2770) after the Asp-N digestion of deglycosylated PrP^{Sc} purified from the R148H-affected subject. Two peptide ions with well-resolved isotopic envelopes were detected, with monoisotopic peaks (arrows) at m/z values of 2731.6 and 2750.5, respectively. **B:** The predicted amino acid sequences, and the theoretical ([M+H]⁺th, calculated) and experimental ([M+H]⁺ex, from **A**) monoisotopic mass values of the wild-type and mutant PrP peptides corresponding to the Asp-N cleavage between PrP residues 147 to 166. The mutation at residue 148 was underlined. Both mutant and wild-type PrP peptides were detected by MALDI-MS.

Figure 4. Lesion profiles. Familial CJD^{R148H} (square, thick line), sCJDMV2 (triangle, thick line), and sCJD MM type 1 (circle, thin line). For sCJDMV2 and sCJDMM1, data represent the sum of three scores (spongiform degeneration, astrogliosis, and neuronal loss) for each brain region examined and expressed as mean $\pm$ SD of three independent experiments. The following gray matter regions were analyzed: FC, TC, PC, OC, HI, EC, BG, TH, PN, and CE.

firmed by the comparative analysis of the lesion profile, which showed that severity and distribution of the histological lesions in various brain regions in fCJD^{R148H} were very similar to those of sCJDMV2 but distinct from those of the common type of sCJDMM1 (Figure 4).³

Gel Mobility Characteristics and Regional Distribution of PrP^{Sc}

After treatment with PK, PrP^{Sc} from sCJD^{R148H} showed the gel mobility of the PrP^{Sc} type 2 (Figure 5).¹⁹ The percentages of distribution of the three glycoforms of PrP^{Sc} from this case identified as diglycosylated, monoglycosylated, and unglycosylated forms (mean $\pm$ SD) were 29 ± 5 , 60 ± 7 , and 11 ± 8 , respectively, similar to the glycoform percentages of distribution of sCJD MV2 that was 39 ± 4 , 54 ± 4 , and 7 ± 3 but slightly different from 22 ± 5 , 53 ± 3 , and $25 \pm 5\%$ distribution of sCJD MM1. Although in both fCJD^{R148H} and sCJDMV2, the diglycosylated form was better represented than or sim-

Figure 5. One-dimensional Western blot of PrP^{Sc}. Brain homogenates from FC of fCJD^{R148H}, sCJDMV2, and sCJDMM1 subtypes were treated with PK (100 μ g/ml) and subjected to SDS-PAGE and immunoblotting with 3F4 mAb.

Figure 6. Quantitative regional distribution of PK-resistant PrP^{Sc}. Familial CJD^{R148H} (square, thick line), sCJDMV2 (triangle, thin line), and sCJDM1 (circle, thin line). The following gray matter regions were examined: FC, OC, HI, EC, BG, TH, PN, and CE. The highest value (BG of fCJD^{R148H}) was arbitrarily rated 100 U, and all of the other values were normalized to 100 and expressed in units. For sCJDMV2 and sCJDM1, data were obtained from three cases and expressed as mean $\pm$ SD. Data from fCJD^{R148H} are means of three independent experiments.

ilar to the unglycosylated form, the opposite was generally observed in sCJDM1.³

The PrP^{Sc} brain distribution was characterized by a relatively low level of PrP^{Sc} in the neocortex, especially in the occipital cortex where bands were barely visible; a higher amount of PrP^{Sc} was present in the caudate nucleus, followed by entorhinal cortex, thalamus, and cerebellum (Figure 6). This distribution was similar to that seen in sCJDMV2. Overall, in fCJD^{R148H}, the amount of PrP^{Sc} was definitely lower than that of sCJDMV2 in cerebral cortical regions but overall fairly similar in the subcortical regions. In both conditions, the PrP^{Sc} distribution differed from that observed in sCJDM1.

PrP Truncated Forms

Because the presence of N-terminally truncated PrP fragments can help in distinguishing different CJD subtypes,^{22,25} we looked for these fragments in S2 and P2 fractions from fCJD^{R148H} and sCJDMV2 using antibodies against various PrP regions. Examination of S2 after PNGase F treatment demonstrated a ~26-kD fragment in fCJD^{R148H} but not in sCJDMV2. This fragment was visible by probing with both 3F4 and anti-C but not anti-N antibody that is against the N terminus of the protein, indicating that the ~26-kD fragment lacks the N-terminal region (Figure 7). However, P2 that contains PrP^{Sc} showed no significant difference in the N-terminally truncated fragments between the R148H mutation and sCJDMV2. Furthermore, both conditions had the C-terminal PrP fragments of 12 and 13 kD (PrP-CTF12/13) (data not shown).^{22,25}

Two-Dimensional Immunoblotting

We further compared insoluble PrP from fCJD^{R148H} and sCJDMV2 on two-dimensional immunoblots (2D blots), which separate proteins with high resolution based not only on the relative mass as the one-dimensional (1D) blot but also on the molecule charges. Six major sets of PrP spots distributed over different pH

Figure 7. N-terminally truncated PrP fragment in S2 fraction from brain homogenate. Immunoblot analysis of PNGase F-treated soluble PrP from fCJD^{R148H} (lane A) and two cases of sCJDMV2 (lanes B and C). PrP was detected with the anti-N, (23 to 40), 3F4 (109 to 112), and anti-C (220 to 231) antibodies. A ~26-kD PrP band (asterisk) in samples from fCJD^{R148H} were visible by probing with 3F4 and the anti-C but not the anti-N antibody, suggesting that this band could be a PrP fragment truncated at the N terminus associated with the R148H phenotype. The blots are representative of at least three independent experiments.

ranges and with different molecular weights were detected in P2 fractions from both fCJD^{R148H} and sCJDMV2 (Figure 8, A and B). The uppermost sets I and II contained diglycosylated and monoglycosylated full-length PrP species and set III unglycosylated full-length PrP species. Sets IV, V, and VI corresponded to N-terminally truncated, diglycosylated, monoglycosylated, and unglycosylated PrP species, respectively. We designated these sets PrP^{ins} 2D spots I to VI (W.Q. Zou and P. Gambetti, unpublished data). When we compared the 2D blots of the insoluble PrP obtained from sCJDMV2 and fCJD^{R148H}, we observed an additional minor set of four to six spots between PrP^{ins} 2D spots I and II (Figure 8A), which may correspond to diglycosylated 26-kD PrP species observed in the 1D blot of S2 but not of P2, probably because of the insufficient resolution of the 1D blot (Figure 7). Furthermore, the PrP^{ins} 2D spots IV was better represented in fCJD^{R148H} than in sCJDMV2, suggesting that there were more truncated forms in fCJD^{R148H} (Figure 8, A and B).

After PK digestion, 2D patterns of PrP27–30 showed less heterogeneity (Figure 8, C and D). The blots from both fCJD^{R148H} and sCJDMV2 were observed to have three sets of PrP spots corresponding to the three N-terminally truncated forms including diglycosylated (IV'), monoglycosylated (V'), and unglycosylated (VI') PrP species. Both di- and mono-glycosylated forms were better represented in the acidic region of the pH gradient in fCJD^{R148H} than in sCJDMV2 preparations consistent with a slight difference in the PrP^{Sc} glycans in the two diseases. This conclusion was supported by the 2D blots of PK-resistant PrP^{Sc} after deglycosylation with PNGase F and probed with the 3F4 antibody, which showed virtually no difference in mobility along the pH gradient between fCJD^{R148H} and sCJDMV2 preparations (2D spots VI'). Two sets of spots were detected. One set contained at least five spots that migrated at about 19 kD (2D spots VI'b); the second that included three to five spots (2D spots VI'a) had a slightly higher relative mass (~0.5 kD) and was underrepresented compared with 2D spots VI'b. The 2D spots VI'a appeared to be more prominent in fCJD^{R148H} than in sCJDMV2 (Figure 8, E and F). Probing the same 2D blots with the anti-C antibody revealed three

Figure 8. Two-dimensional Western blot analyses of P2 fraction from FC. Familial CJD^{R148H} and sCJDMV2 samples were subjected to isoelectric focusing (IEF), SDS-PAGE, and immunoblotting with mAb 3F4 (**A–F**) or anti-C antibody (**G** and **H**). **A, C, E,** and **G:** fCJD^{R148H}; **B, D, F,** and **H:** sCJDMV2. **A** and **B:** Untreated samples; **C** and **D:** PK-treated samples; **E** to **H:** PK + PNGase F-treated samples. The small inset in **A** is magnification of the PrP 2D spots I'. The blots are representative of at least three independent experiments and at least three distinct cases of sCJDMV2.

additional sets of spots with gel mobilities at about 17 to 18 kD (VII), 12 to 13 kD (VIII), and 7 to 8 kD (IX), respectively (Figure 8, G and H).^{22,25} They represented PrP fragments truncated at various sites of the N terminus. Although these fragments were present in both fCJD^{R148H} and sCJDMV2, their representation on the blots appeared different. The 19- and 12- to 13-kD species were predominant in fCJD^{R148H}, whereas in sCJDMV2 the 7- to 8-kD PrP species was at least as well represented as the 19-kD species.

Figure 9. Conformational stability assay of PrP^{Sc}. **A** and **B:** Western blots of PrP_{27–30} recovered after treatment with increasing concentrations of GdnHCl. Aliquots of brain homogenates from FC of fCJD^{R148H} (**A**) and sCJDMV2 (**B**) were treated with various concentrations of GdnHCl at room temperature for 1 hour. GdnHCl was subsequently removed by precipitation, and PK digestion was performed before Western blotting with mAb 3F4. **C:** The levels of PrP_{27–30} from fCJD^{R148H} (◆) and sCJDMV2 (■) as a function of increasing concentrations of GdnHCl. Data obtained by densitometry of Western blots, as shown in **A** and **B**, are the average of independent experiments with three fCJD^{R148H} samples from this case and six sCJDMV2 cases (mean ± SD).

Conformational Stability Assay of PrP^{Sc}

This assay has been used to characterize the conformation of PrP^{Sc} as a function of the concentration of GdnHCl needed to render PrP^{Sc} PK sensitive (Figure 9).^{12,13,23} The GdnHCl concentration required to make one-half of the PrP^{Sc} PK sensitive (GdnHCl 1/2) is used as a measurement of the relative conformational stability of PrP^{Sc}. The GdnHCl 1/2 was $\sim 1.58 \pm 0.03$ mol/L ($n = 3$) for PrP^{Sc} from fCJD^{R148H} and $\sim 1.81 \pm 0.21$ mol/L ($n = 6$) for PrP^{Sc} from sCJDMV2 (Figure 9, A–C), respectively; the difference was not statistically significant ($P = 0.11$, two-tailed Student's *t*-test), indicating that there was no significant difference in the conformational stability between the two PrP^{Sc} species.

Discussion

The first possibility that should be ruled out when encountering a new mutation, especially like the R148H mutation that is associated with a phenotype mimicking sCJD and has no history of familial disease, is whether the gene variation represents a mutation rather than a rare polymorphism. The R148H variation has apparently never been observed after sequencing of several thousand *PRNP* alleles in normal populations.^{6,26,27} In contrast, it has been observed to be associated with a prion disease in two cases: our case and that of Krebs et al.⁶ Furthermore, the immunoblot of the Krebs et al case demonstrated a PK-treated PrP^{Sc} that had a different gel mobility

from that of the PK-treated PrP^{Sc} associated with the corresponding sCJD, indicating that the PrP^{Sc} conformation is affected by the R148H variation.⁶ These considerations argue that the R148H variation is a mutation rather than a rare polymorphism. Because in both our case and the case of Krebs et al, there were no other affected family members, it is likely that the R148H mutation has low penetrance or when it occurs in subjects homozygous methionine at codon 129, as in the sisters of the proband, is associated with a very late disease onset so that carriers may escape fCJD^{R148H} due to earlier death from other causes.

The R148H mutation is of interest in at least three aspects. First, it is the only point mutation that is located in the α -helix 1 region of PrP.^{4–6} Second, it is associated with a histopathological phenotype that has never been reported in fCJD, yet it is indistinguishable from the phenotype of a subtype of sCJD identified as sCJDMV2.^{3,5,19} Third, compared with the case of fCJD^{R148H} of Krebs et al,⁶ it further emphasizes the role of the polymorphism at codon 129 in determining the disease phenotype.

Studies with nuclear magnetic resonance have demonstrated that human recombinant PrP, encompassing residues 23 to 230, has an N-terminal disordered “tail” and a C-terminal globular domain.²⁸ At neutral pH, the latter domain includes two short β -strands (residues 128 to 131 and 161 to 164) and three α -helices that are composed of residues 144 to 156, 173 to 194, and 200 to 228, respectively.^{28,29} The α -helix 1 is believed by most investigators to play a critical role in the conversion of PrP^C into PrP^{Sc} as the PrP^C conformational change leading to the formation of an expanded β -strand region would take place in the α -helix 1 and would also involve the two β -strand domains; the remaining two α -helices are conserved.^{30–32} The R148H is the first point mutation to be observed in α -helix 1. By replacing the R with an H in the mid-portion of this helix, the R148H mutation likely weakens at least one of the two salt bridges that are located in α -helix 1 and that are believed to stabilize PrP^C from converting to PrP^{Sc}.³²

The finding that the disease phenotype in the present case of fCJD^{R148H} is virtually indistinguishable from that of sCJDMV2, a distinct type of sCJD, despite the different etiology of the two diseases, offers the opportunity to examine the issues of relationships between phenotypic and PrP^{Sc} characteristics. Familial prion diseases include three major disease phenotypes: CJD, fatal familial insomnia (FFI), and Gerstmann-Sträussler-Scheinker disease.⁴ Although at least 15 point mutations of *PRNP*, including R148H, are associated with the CJD pathological phenotype, the number of distinct disease phenotypes associated with these mutations is relatively limited.^{4–6} Five of the seven mutations in which the phenotype has been characterized reproduce the phenotype associated with the sCJDM1, the classical type of sporadic CJD.⁴ One mutation appears to imitate the phenotype associated with sCJDV1, a rare type of sporadic CJD, whereas another one is somewhat distinct from the known forms of sporadic CJD.⁴ The frequent matching of the phenotypes associated with familial and sporadic CJD has prompted the suggestion that the phenotypes of

fCJD imitate those of sCJD, thus arguing that despite their different etiology, sporadic and familial forms share the same PrP^{Sc} characteristics.^{8,9}

The R148H mutation seems to follow this principle because the disease phenotype associated with it is virtually indistinguishable from the phenotype associated with sCJDMV2.^{3,5,19} The histopathology is very similar in the two diseases as far as the distribution, type, and severity of the lesions; PrP^{Sc} also is of the same type, is similarly distributed in the brain regions, and has the same glycoform ratio. These findings suggest that the PrP^{Sc} species present in fCJD^{R148H} and in sCJDMV2 share the same characteristics and belong to the same strain even if one PrP^{Sc} species is allegedly the result of a pathogenic mutation in *PRNP* and the other presumably results from a random and spontaneous conformational change of PrP^C. Here, we investigated this possibility and attempted to gain insight into the relationship between PrP^{Sc} strain and disease phenotype by further assessing the similarity of these two PrP^{Sc} species. A combination of structure mapping of PrP by various anti-PrP antibodies and MALDI mass spectrometry, 2D immunoblotting, and CSA allows for a finer evaluation of the physicochemical characteristics of PrP^{Sc}.

On 1D blots, only one difference was found in S2, which presumably contains both PrP^{Wt} and PrP^M in fCJD^{R148H} preparations and PrP^{Wt} in the preparations from sCJDMV2. This difference was the presence in fCJD^{R148H} of a PrP fragment that is truncated at the N terminus. After de-glycosylation, this PrP fragment migrated to ~26 kd (Figure 7) and to a highly acidic region (pH ~3.5) on 2D immunoblots (data not shown). Although this fragment was not detectable on 1D blots of P2, the fragment appeared to be present on the 2D blots. Only minor differences between fCJD^{R148H} and sCJDMV2 were detected on the 2D blots of the PK-untreated detergent insoluble preparations containing full-length PrP^{Sc}, as well as in the PK-treated PrP^{Sc} fractions that only contained the PK-resistant PrP^{Sc} fragments. Mobility along the pH gradient, particularly of the PK-treated PrP^{Sc} glycosylated forms, was slightly different between the two preparations (Figure 8, C and D, sets IV and V). This likely reflects the diversity in the glycans associated with the two PrP^{Sc} species, resulting in PrP^{Sc} glycoforms with different charges and thus revealing different isoelectric points (pI).

The other two differences are quantitative rather than qualitative and relate to the different representations of the N-terminally truncated forms. First, after removal of the glycans, the major PrP27–30 migrates to about 19 kd in two rows of spots with similar but not identical gel mobility (Figure 8, E and F). Although the spots that migrate to the lower row (VI'b) appear to be similar in number for the two diseases, those in the upper row (VI'a) appear more prominent in fCJD^{R148H} than in sCJDMV2. The PK cleavage of PrP^{Sc} associated with sCJDMV2 is ragged. The most common cleavage site is at residue 97; however, other relatively well-represented cleavages occur at residues 92 and 82.¹⁸ Therefore, it is likely that VI'b represents the PrP^{Sc} fragment generated by the PK cleavage at residue 97. VI'a then likely repre-

sents the fragment cleaved at residue 92. Should this be the case, more PrP^{Sc} isoforms are cleaved by PK at a more N-terminal site in fCJD^{R148H} than in sCJDMV2.

Second, examination of all of the forms of the N-terminally truncated PrP^{Sc} generated by PK treatment revealed the same three sets of fragments in fCJD^{R148H} and sCJDMV2 preparations. These include fragments of approximate size 17 to 18 kd, 12 to 13 kd, and 7 to 8 kd. Representations of these fragments, however, are different. The 19-kd and 12- to 13-kd fragments are more prominent in fCJD^{R148H}, whereas the opposite is true for the 17- to 18-kd and 7- to 8-kd fragments. The finding that the PK-cleavage sites are identical in the PrP^{Sc} associated with fCJD^{R148H} and sCJDMV2, yet are represented differently in the two PrP^{Sc} species, argues that the conformation of PrP^{Sc} in fCJD^{R148H} is slightly different from that of PrP^{Sc} associated with sCJDMV2 and thus favors a secondary cleavage site by PK.

CSA of PrP^{Sc}, a modification of the conformation-dependent immunoassay originally introduced by Safar et al,¹² is thought to be a sensitive test for the detection of conformational characteristics that can distinguish different prion strains.^{12–14} The assay is based on the principle that differences in protein structure and aggregation can be measured by the rate in which the protein unfolds after increasing levels of chemical denaturation. CSA has been used previously to measure the unfolding rate of PrP^{Sc} denatured by GdnHCl, revealing a decrease in the amount of PrP27–30 remaining in the preparation.^{12–14,23} Peretz et al^{13,14} showed that the PrP^{Sc} species with similar CSA values cause diseases with comparable phenotypic characteristics, although occasionally similar conformational stability values have been observed in PrP^{Sc} associated with distinct phenotypes. Here, we used CSA to explore whether or not fCJD^{R148H} and sCJDMV2 that share the same phenotype also have similar PrP^{Sc} strains despite the different etiology. The GdnHCl_{1/2} of PrP^{Sc} from fCJD^{R148H} is different from that of PrP^{Sc} from sCJDMV2 (1.58 ± 0.03 versus 1.82 ± 0.21 mol/L); however, this difference failed to indicate any statistical significance ($P = 0.11$). In contrast, the GdnHCl_{1/2} values of PrP^{Sc} obtained from minks with “drowsy” or “hyper” transmissible encephalopathies that have distinct phenotypes and PrP^{Sc} species, which we used as control, were significantly different (data not shown).

In addition, we examined whether PrP27–30 present in fCJD^{R148H} included both PrP^M and PrP^{Wt} species. This is relevant to the present comparative study because it has been shown that, at least in one case of sCJDMV with PrP^{Sc} type 1, PrP27–30 involved both 129M and 129V PrP allotypes (M. Pocchiari, personal communication).³³ It is reasonable to assume that the two allotypes also participate in the formation of PrP^{Sc} in sCJDMV2. Likewise, we observed a similar result in fCJD^{R148H}. Using MALDI-mass spectrometry, both PrP allotypes, ie, PrP^M-129M and PrP^{Wt}-129V, were found in PrP27–30 purified from fCJD^{R148H}. Therefore, the PrP^{Sc} molecule from the two conditions is most likely to be made up of both 129M and 129V alleles. This finding adds one more similarity to PrP^{Sc} associated with the two diseases.

At variance with our case (designated fCJD^{R148H}-129MV), the subject reported by Krebs et al⁶ was homozygous for M at codon 129 (designated fCJD^{R148H}-129MM). Remarkably, the phenotypic and PrP^{Sc} characteristics, although briefly reported, basically appear to be different from those we observed in our fCJD^{R148H}-129MV. The phenotype of fCJD^{R148H}-129MM mimicked that of the typical sporadic CJD also identified as sCJDM1 in the clinical features, histological lesions, and pattern of PrP immunostaining.^{3,6,19} Neither kuru plaques nor the plaque-like PrP immunostaining pattern was observed in fCJD^{R148H}-129MM. PrP^{Sc} was not of type 2 as in our cases, but it had unusual gel mobility with the unglycosylated form of PrP27–30 migrating to approximately 22 kd, about 1 kd slower than PrP^{Sc} type 1.^{6,19}

The genotype of the polymorphic codon 129 is a well-known modifier of the disease phenotype in sporadic and familial prion diseases.^{1,4} In familial prion diseases, a striking example has been provided by fCJD^{D178N} and FFI, two phenotypically distinct diseases associated with PrP^{Sc} type 1 and 2, respectively.^{7,15} Both fCJD^{D178N} and FFI are linked to the same D178N mutation but to different genotypes at the codon 129 MV polymorphism coupled with the D178N mutation. The result is the formation of two distinct haplotypes: D178N-129V in fCJD^{D178N} and D178N-129M in FFI.⁷ Therefore, the codon 129 aligned with the D178N mutation modifies basic phenotypic features of the disease. Furthermore, depending on the codon 129 of PrP^{Wt}, fCJD^{D178N}-affected subjects can be homozygous for V, and FFI-affected subjects can be homozygous for M; both patient populations can be M or V heterozygous. In both diseases, the course is shorter in 129 homozygous than in heterozygous. In FFI, a disease that has been studied more extensively than fCJD^{D178N}, some of the clinical signs and histopathology may be slightly different between 129 homozygous and heterozygous subjects, but the major characteristics of PrP^{Sc} are the same.⁴ Therefore, codon 129 on the PrP^{Wt} allele modulates minor features within the phenotype determined by the mutated haplotype. Similar findings have been reported in other *PRNP* mutations.⁴ In contrast, the striking differences in the phenotypic and PrP^{Sc} characteristics between fCJD^{R148H}-129MV and fCJD^{R148H}-129MM suggest the novel conclusion that in the R148H mutation, codon 129 can modify basic phenotypic and PrP^{Sc} features even when located on the normal allele.

In conclusion, our comparative study of phenotypic and PrP^{Sc} characteristics associated with fCJD^{R148H} and sCJDMV2 reveals that the disease phenotype is indistinguishable in these familial and sporadic forms of CJD. Furthermore, all major features of PrP^{Sc}, such as the type, brain distribution, and the glycoform ratio, are identical, whereas other more sensitive measurements such as 2D immunoblotting and CSA show only minor or statistically insignificant differences. These findings argue that at variance with other *PRNP* point mutations that are often associated with distinctive changes in PrP^{Sc}, the R148H mutation seems to trigger a prion disease that mimics virtually all of the features of a sporadic form of CJD. Therefore, it is tempting to suggest that despite the different modalities and perhaps tempos in which they oc-

cur, the spontaneous conversion of PrP^{Wt} in sCJD and of PrP^M in fCJD, into PrP^{Sc} may lead to the formation of similar prion strains that, in turn, result in the expression of similar disease phenotypes. Furthermore, the comparative study of the first two cases of fCJD^{R148H} provides a novel mechanism by which the *PRNP* codon 129 can affect the expression of principal phenotypic and PrP^{Sc} features also when it is located on the normal allele.

Acknowledgments

We thank Dr. Richard Bessen (Department of Veterinary Molecular Biology, Montana State University of Bozeman) for providing prion-infected mink brain tissues and Diane Kofskey and Phyllis Scalzo for technical support in studies of histology and immunohistochemistry.

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