

ORIGINAL ARTICLE

Extraneural Pathologic Prion Protein in Sporadic Creutzfeldt–Jakob Disease

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ABSTRACT

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BACKGROUND

In patients with sporadic Creutzfeldt–Jakob disease, pathologic disease-associated prion protein (PrP^{Sc}) has been identified only in the central nervous system and olfactory-nerve tissue. Understanding the distribution of PrP^{Sc} in Creutzfeldt–Jakob disease is important for classification and diagnosis and perhaps even for prevention.

METHODS

We used a highly sensitive method of detection — involving the concentration of PrP^{Sc} by differential precipitation with sodium phosphotungstic acid, which increased the sensitivity of Western blot analysis by up to three orders of magnitude — to search for PrP^{Sc} in extraneural organs of 36 patients with sporadic Creutzfeldt–Jakob disease who died between 1996 and 2002.

RESULTS

PrP^{Sc} was present in the brain tissue of all patients. In addition, we found PrP^{Sc} in 10 of 28 spleen specimens and in 8 of 32 skeletal-muscle samples. Three patients had PrP^{Sc} in both spleen and muscle specimens. Patients with extraneural PrP^{Sc} had a significantly longer duration of disease and were more likely to have uncommon molecular variants of sporadic Creutzfeldt–Jakob disease than were patients without extraneural PrP^{Sc}.

CONCLUSIONS

Using sensitive techniques, we identified extraneural deposition of PrP^{Sc} in spleen and muscle samples from approximately one third of patients who died with sporadic Creutzfeldt–Jakob disease. Extraneural PrP^{Sc} appears to correlate with a long duration of disease.

PRION DISEASES ARE CHARACTERIZED BY degeneration of central nervous tissue associated with the replication of a transmissible agent, a prion.¹ Prions are mainly composed of an abnormal, partially protease-resistant conformer (PrP^{Sc}) of a cellular protein (PrP^C).² Although tissue damage occurs only in the central nervous system, the accumulation of PrP^{Sc} and prion infectivity are not necessarily confined to neural tissue in all prion diseases. In scrapie in sheep, chronic wasting disease in deer, and numerous animal models of prion diseases, prions invade the lymphoreticular system.³ PrP^{Sc} has also been reported in the skeletal muscle of mice with experimentally induced prion disease.⁴

The most common prion disease in humans is Creutzfeldt-Jakob disease, which has been classified as sporadic, familial, iatrogenic, or variant. The causation of sporadic Creutzfeldt-Jakob disease is unclear, whereas biochemical, histopathological, and epidemiologic evidence suggests that the variant form results from the transmission of bovine spongiform encephalopathy prions to humans.^{5,6} PrP^{Sc} accumulates in tonsils⁷ and other lymphoreticular organs⁸ of patients with variant Creutzfeldt-Jakob disease,⁹ whereas the sporadic form of the disease is not thought to target the lymphoreticular system.¹⁰ The allegedly unique involvement of the lymphoreticular system in variant Creutzfeldt-Jakob disease serves as a diagnostic criterion to distinguish variant from sporadic Creutzfeldt-Jakob disease and is one of the principal pieces of evidence that these forms represent two distinct disease entities.⁶ Refinements in the sensitivity of the methods of detection¹¹ prompted us to reinvestigate the distribution of PrP^{Sc} in humans with sporadic Creutzfeldt-Jakob disease.

METHODS

COLLECTION OF SAMPLES

We collected central nervous system, spleen, and muscle samples at autopsy from a cohort of Swiss patients who died between 1996 and 2002: 36 patients with prion disease (36 brain specimens, 32 muscle specimens, and 28 spleen specimens), 10 with Alzheimer's disease (10 muscle and 8 spleen samples), and 9 patients without neurologic disorders (9 muscle and 9 spleen samples). The utmost care was taken to avoid contamination of extraneural samples with central nervous system tissue. All

tissues were processed according to established guidelines regarding safety and ethics.

DETECTION OF PrP^{Sc} IN EXTRANEURAL TISSUES

All procedures were carried out in biosafety level 3 facilities with strict adherence to safety guidelines. The method of precipitation with the use of sodium phosphotungstic acid was adapted from published protocols.⁸ We prepared 10 percent tissue homogenates (weight per volume) in 2 percent sarkosyl in sterile phosphate-buffered saline using a RiboLyser (Hybaid). Cellular debris was removed by centrifugation at 80×g for one minute in a microcentrifuge. Occasional residual debris was removed by additional centrifugation at 2700×g for five minutes. Supernatants (500 μl) were mixed with equal volumes of 2 percent sarkosyl in phosphate-buffered saline and incubated for 10 minutes at 37°C. Benzonase (Benzon nuclease, Merck) and magnesium chloride were added (final concentration, 50 units per milliliter and 1 mmol per liter, respectively), and the mixture was incubated under constant agitation for 30 minutes at 37°C.

Samples were adjusted to a final concentration of 0.3 percent phosphotungstate, incubated at 37°C for 30 minutes under constant agitation, and centrifuged at 15,800×g for 30 minutes in a microcentrifuge. The pellets were resuspended in 20 μl of phosphate-buffered saline with 0.1 percent sarkosyl. Samples were adjusted to a final concentration of 20 μg of proteinase K per milliliter and incubated at 37°C for 30 minutes. Digestion was terminated by the addition of protease inhibitors (Complete protease inhibitor cocktail, Boehringer Mannheim). Samples were boiled in loading buffer (125 mM TRIS sodium chloride, 4 percent sodium dodecyl sulfate, 20 percent glycerol, and 0.02 percent bromophenol blue), subjected to electrophoresis in 12 percent TRIS-glycine gels, and blotted on nitrocellulose membranes. Membranes were blocked with 5 percent Topblock (Jura) in phosphate-buffered saline with 0.05 percent Tween and then incubated overnight with a monoclonal antibody against PrP (3F4; dilution 1:5000).¹²

The samples were then incubated with an alkaline-phosphatase-conjugated secondary antibody (1:20,000) and examined with use of 2-chloro-5-(4-methoxy Spiro[1,2-dioxetan-3,2'-(5'-chlor)tricycl[3.3.1.1^{3,7}]decan]-4-yl)-1-phenylphosphate dinatrium salt chemiluminescent substrate (Amersham) and a VersaDoc digital imager (model 5000,

Bio-Rad). Each blot included appropriate positive and negative controls (10 μ g or 100 μ g of brain homogenate from a patient with sporadic Creutzfeldt–Jakob disease or a patient without a prion disease added to 50 mg of muscle or spleen specimen from a patient without a prion disease). All analyses were carried out and all but six samples were examined at least twice by independent investigators. Samples were deemed positive if proteinase K–resistant diglycosylated, monoglycosylated, and unglycosylated bands with electrophoretic motility indicative of PrP^{Sc} were unambiguously identified.

DETECTION OF PrP^{Sc} IN NEURAL TISSUES

Western blot analysis was carried out as described previously.¹³ PrP glycoforms were quantified with use of a Kodak image station (model 440) or a Versa-Doc digital imager (model 5000), and patients were typed according to the size of the protease-resistant core PrP fragment¹⁴ and the prevalence of glycoforms.^{15,16}

GENETIC ANALYSIS

Genomic DNA was extracted from frozen tissues. Polymerase chain reaction and analysis of the entire coding region of the prion protein gene (PRNP) were performed with use of standard techniques and software (SeqScape, Applied Biosystems). In addition, codon 129 polymorphisms were identified by restriction-fragment–length polymorphism analysis.¹⁷

HISTOLOGIC ANALYSIS

Tissue was fixed with 4 percent buffered formalin, inactivated by exposure to 98 percent formic acid for one hour, and embedded in paraffin. Sections (3 μ m) were subjected to conventional staining and to immunostaining for glial fibrillary acid protein (Dako) and PrP (monoclonal antibody 3F4) after hydrolytic autoclaving.

RESULTS

We analyzed a total of 45 spleen specimens and 51 skeletal-muscle samples from 36 patients with histopathologically, biochemically, and genetically confirmed sporadic Creutzfeldt–Jakob disease (mean [\pm SD] age, 66.8 \pm 8.7 years; 20 men and 16 women); 10 patients with Alzheimer's disease (mean age, 76.2 \pm 4.6 years; 6 men and 4 women); and 9 patients without neurologic disease (mean age, 59 \pm 19.2 years; 5 men and 4 women). The age distribution,

sex ratio, and duration of disease (mean, 5.0 \pm 4.6 months) of the cohort of patients with sporadic Creutzfeldt–Jakob disease did not differ significantly from those in published series.¹⁸

ACCUMULATION OF PrP^{Sc} IN SPLEEN AND SKELETAL MUSCLE

Western blot analysis of protease-resistant prion protein after phosphotungstate precipitation showed PrP^{Sc} in 10 of 28 spleen samples and in 8 of 32 skeletal-muscle samples from the patients with sporadic Creutzfeldt–Jakob disease (Fig. 1). Three patients had PrP^{Sc} in both spleen and muscle (Table 1). Assays were repeated with independently homogenized tissue fragments: three spleen and three muscle samples always tested positive, whereas others yielded variable results (Table 1), possibly because of inhomogeneous peripheral distribution of PrP^{Sc}, as described previously.^{4,19}

Control samples of spleen and muscle were assessed in parallel: none of them contained PrP^{Sc} (data not shown). Patients with PrP^{Sc} in spleen and patients without PrP^{Sc} in spleen did not differ significantly with respect to age (Table 2). However, patients with PrP^{Sc} only in spleen or in spleen or muscle or both had significantly longer intervals between the onset of clinical symptoms and death than did patients without extraneural PrP^{Sc}. Patients with PrP^{Sc} in muscle tended to be younger at death than patients without PrP^{Sc} in muscle (Table 2). None of the subgroups differed significantly with respect to the ratio of men to women.

One patient with splenic PrP^{Sc} had received a cadaveric dura mater transplant more than 22 years before the onset of dementia. This case was deemed unlikely to represent iatrogenic transmission, since the incubation period would be longer than that in any other documented case of dura mater–associated transmission.²⁰

GENETIC ANALYSIS OF PRNP

We sequenced the entire open reading frame of PRNP from all patients with extraneural PrP^{Sc} and found no disease-associated mutations. The common methionine–valine polymorphism at codon 129 was analyzed in all 10 patients with splenic PrP^{Sc} and all 8 patients with muscle PrP^{Sc} as well as in 15 patients without splenic PrP^{Sc} and 21 patients without muscle PrP^{Sc}. We observed a tendency toward overrepresentation of heterozygosity for methionine and valine and homozygosity for valine among patients with splenic PrP^{Sc}, whereas the ma-

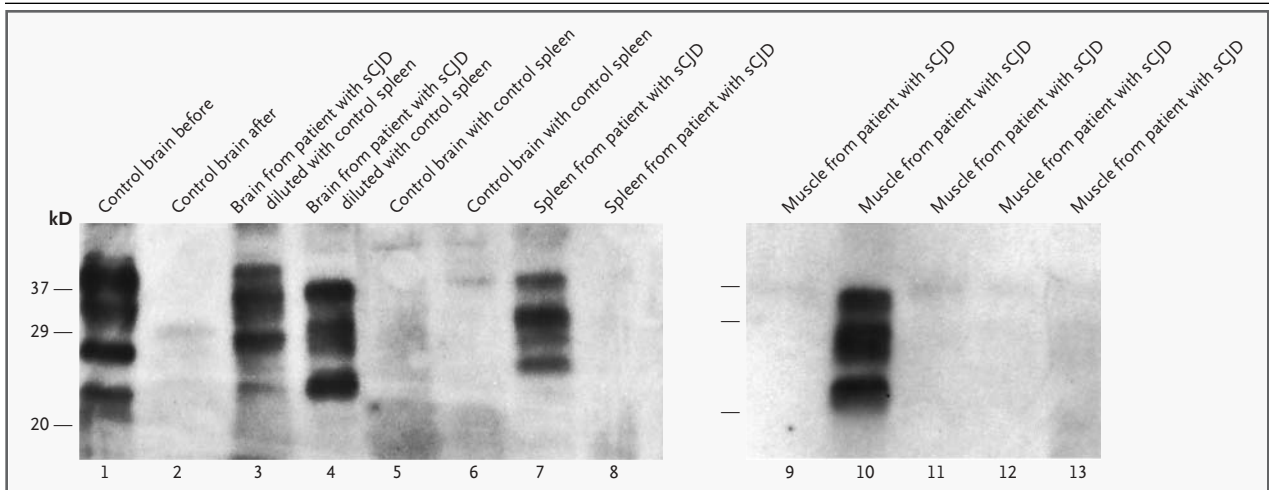


Figure 1. Western Blot Analysis of Phosphotungstate-Precipitated Pathologic Prion Protein (PrP^{Sc}) from Spleen and Muscle Samples from Patients with Sporadic Creutzfeldt-Jakob Disease (sCJD).

Lanes 1 and 2 show Western blot (total protein) analysis of 50 µg of brain homogenate from a patient without Creutzfeldt-Jakob disease. Lanes 3 and 4 show brain homogenate from a patient with sporadic Creutzfeldt-Jakob disease diluted with spleen homogenate from a patient without a prion disease (control); lanes 5 and 6 brain homogenate from a control diluted with spleen homogenate from a control; lanes 7 and 8 spleen homogenate from a patient with sporadic Creutzfeldt-Jakob disease; and lanes 9, 10, 11, 12, and 13 muscle homogenate from a patient with sporadic Creutzfeldt-Jakob disease. PrP^{Sc} is present in two samples (lanes 7 and 10). Lanes 2, 4, and 6 through 13 show results after proteinase K digestion.

majority of patients with muscle PrP^{Sc} were homozygous for methionine (Table 2).

HISTOLOGIC AND BIOCHEMICAL ANALYSES

Typical histopathological features of sporadic Creutzfeldt-Jakob disease, consisting of spongiform changes, neuronal loss, and gliosis, were present in all patients (Fig. 2). The electrophoretic mobility of unglycosylated, protease-digested PrP^{Sc} (also called the core fragment size, which can be 21 kD for type 1 or 19 kD for type 2)¹⁴ allows for the stratification of Creutzfeldt-Jakob disease into subgroups with specific clinical features, which may be caused by different prion strains.⁶ A finer degree of stratification is attained by including information on the codon 129 genotype and patterns of cerebral deposition of PrP^{Sc}; accordingly, we assigned each patient in our series to one of seven proposed groups.¹⁸ This analysis indicated an overrepresentation of uncommon variants of sporadic Creutzfeldt-Jakob disease such as MM2C (homozygous for methionine, type 2, with cortical deposition) and MV2 (heterozygous, type 2) in patients with extraneural PrP^{Sc} (Table 2), suggesting that peripheral deposition of PrP^{Sc} may identify a biochemically or genetically unique subgroup of patients.

Table 1. Age at Onset and Duration of Disease in 15 Patients with Sporadic Creutzfeldt-Jakob Disease and Extraneural Pathologic Prion Protein (PrP^{Sc}).

Patient No.	Age	Duration of Disease	Location of Extraneural PrP ^{Sc}	Muscle*	Spleen*
	yr	mo		no. with PrP ^{Sc} /total no.	
1	47	7	Spleen	0/2	1/4
2	48	6	Muscle	2/2	ND
3	52	6	Spleen	ND	1/2
4	55	8	Spleen	0/2	1/3
5	60	5	Spleen and muscle	1/2	1/1
6	62	3	Muscle	1/2	0/1
7	65	29	Muscle	1/3	ND
8	66	3	Muscle	1/2	0/2
9	67	7	Spleen and muscle	1/2	3/3
10	68	4	Muscle	2/2	0/2
11	68	7	Spleen	0/2	2/4
12	73	5	Spleen and muscle	1/1	2/2
13	77	3	Spleen	0/2	2/3
14	77	4	Spleen	0/2	1/3
15	81	5	Spleen	0/2	1/3

* Up to four individual fragments of each organ were independently homogenized, subjected to phosphotungstate precipitation, and analyzed by Western blotting. Variability in detection may be due to inhomogeneous distribution of PrP^{Sc} in extraneural organs. ND denotes not done.

Table 2. Demographic Characteristics and Classification of Sporadic Creutzfeldt–Jakob Disease in Patients with and Those without Pathologic Prion Protein (PrP^{Sc}) in Spleen and Muscle Specimens.*

Status of Peripheral PrP ^{Sc}	Disease Phenotype†							Sex M/F	Age yr	Duration of Disease mo
	MM1	MV1	VV1P	MM2C	MM2T	MV2	VV2			
	no. of patients									
Spleen sample										
PrP ^{Sc} present	3	1	1	1		2	2	5/5	65.9±6.9	5.7±1.6‡
PrP ^{Sc} absent§	12	1		1		1		11/7	67.9±6.9	4.0±2.2‡
Muscle sample										
PrP ^{Sc} present	5			2			1	4/4	63.6±7.4	7.7±8.7
PrP ^{Sc} absent§	14	2	1			3	1	15/9	67.6±8.8	4.4±2.3
Spleen or muscle sample										
PrP ^{Sc} present	7	1	1	2		2	2	7/8	64.4±10.5	6.8±6.3¶
PrP ^{Sc} absent§	15	1				1		13/8	68.6±7.0	3.8±2.2¶

* Plus–minus values are means ±SD. Data on classification are from Parchi et al.^{14,18} Mann–Whitney tests were used for all comparisons of age and the duration of illness. Sex ratios were compared with use of chi-square tests. Only significant differences between groups are indicated.

† Patients were classified according to the size of the protease-resistant fragment of PrP^{Sc} (type 1 [21 kD] or type 2 [19 kD]), whether they were homozygous for methionine (MM1, MM2C, or MM2T) or valine (VV1P or VV2) at codon 129 of the prion gene or were heterozygous (MV1 or MV2), and histologic characteristics such as plaque or plaque-like lesions (P), cortical or synaptic deposits (C), and thalamic deposits (T).

‡ P=0.004.

§ Patients with no available information on codon 129 were not classified.

¶ P=0.003.

Quantification of the relative prevalence of diglycosylated, monoglycosylated, and unglycosylated PrP^{Sc} is a further instrument for the biochemical characterization of PrP^{Sc}, which allows one to discriminate sporadic from variant Creutzfeldt–Jakob disease.¹⁶ This analysis did not uncover any unorthodox PrP^{Sc} glycotypes in patients with peripheral PrP^{Sc} (Fig. 3): the distribution of brain glycotypes in patients with peripheral PrP^{Sc} was similar to that in other cohorts of patients with sporadic Creutzfeldt–Jakob disease.¹³

DISTRIBUTION OF PrP^{Sc} IN SKELETAL-MUSCLE GROUPS

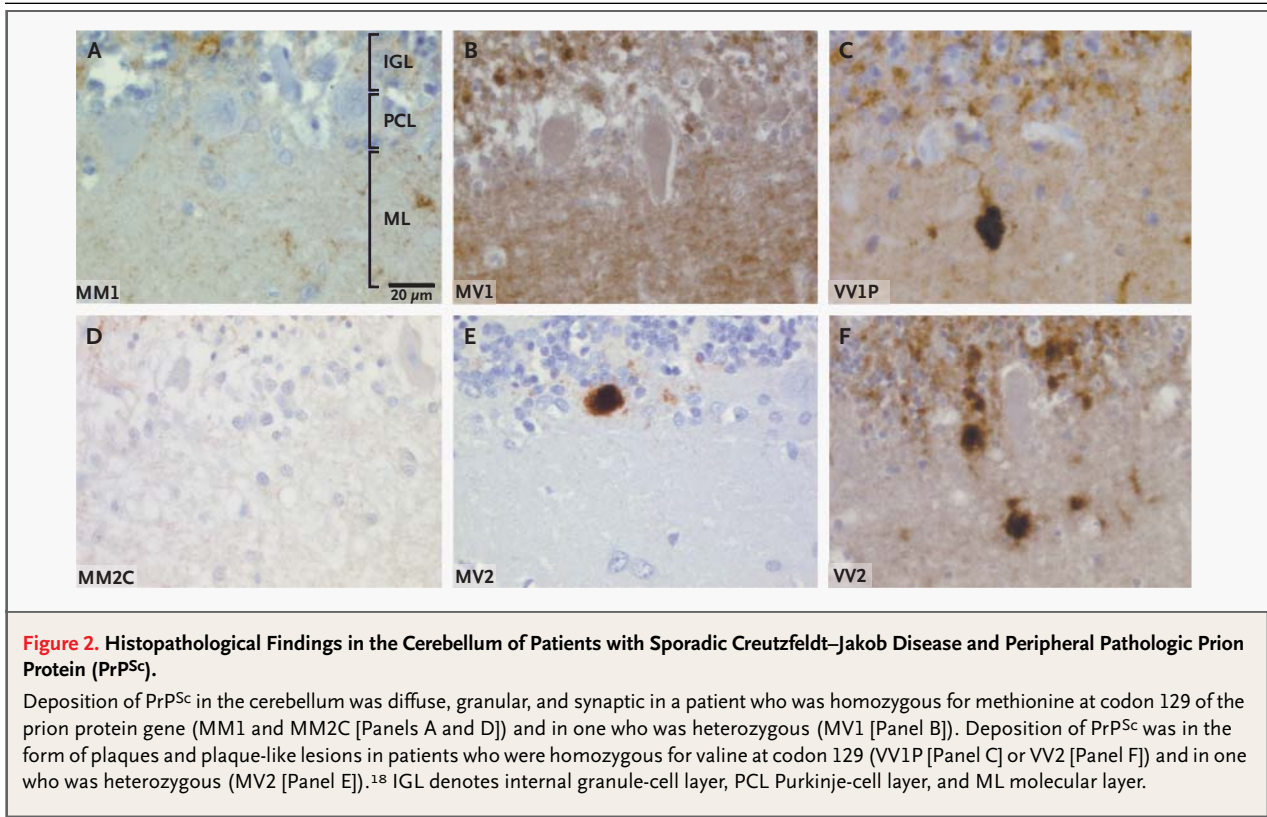
Earlier studies in mice have shown that the PrP^{Sc} content may vary among muscle groups.⁴ In our series, the presence of PrP^{Sc} was not limited to specific muscle groups: PrP^{Sc} was found in four samples of pectoral muscle, two samples of psoas muscle, and two samples of intercostal muscle. It was absent in 10 pectoral-muscle specimens, 5 psoas-muscle specimens, 2 biceps-muscle specimens, and 4 intercostal-muscle specimens. In three patients without extraneural PrP^{Sc}, no information on the anatomical origin of muscle samples could be obtained.

QUANTIFICATION OF PrP^{Sc} IN EXTRANEURAL ORGANS

In order to estimate the relative concentration of PrP^{Sc} in extraneural organs, we compared the intensity of proteinase K–digested Western blot signals with calibration curves obtained by diluting brain homogenate from a patient with Creutzfeldt–Jakob disease with muscle or spleen homogenate from a patient without a prion disease. Despite considerable variations in individual spleen and muscle specimens, the signal intensity of peripheral PrP^{Sc} was generally similar to that obtained when 10 µg of brain homogenate from a patient with sporadic Creutzfeldt–Jakob disease was diluted with 50 mg of spleen or muscle from a patient without a prion disease (Fig. 1). Therefore, we estimate that the levels of PrP^{Sc} in extraneural organs are lower by a factor of approximately 1×10^{-4} than those typically found in the central nervous system of patients with sporadic Creutzfeldt–Jakob disease.

DISCUSSION

Previous studies did not detect PrP^{Sc} in the tonsils, spleen, lymph nodes,¹⁰ or appendix of patients with sporadic Creutzfeldt–Jakob disease.²¹ How-



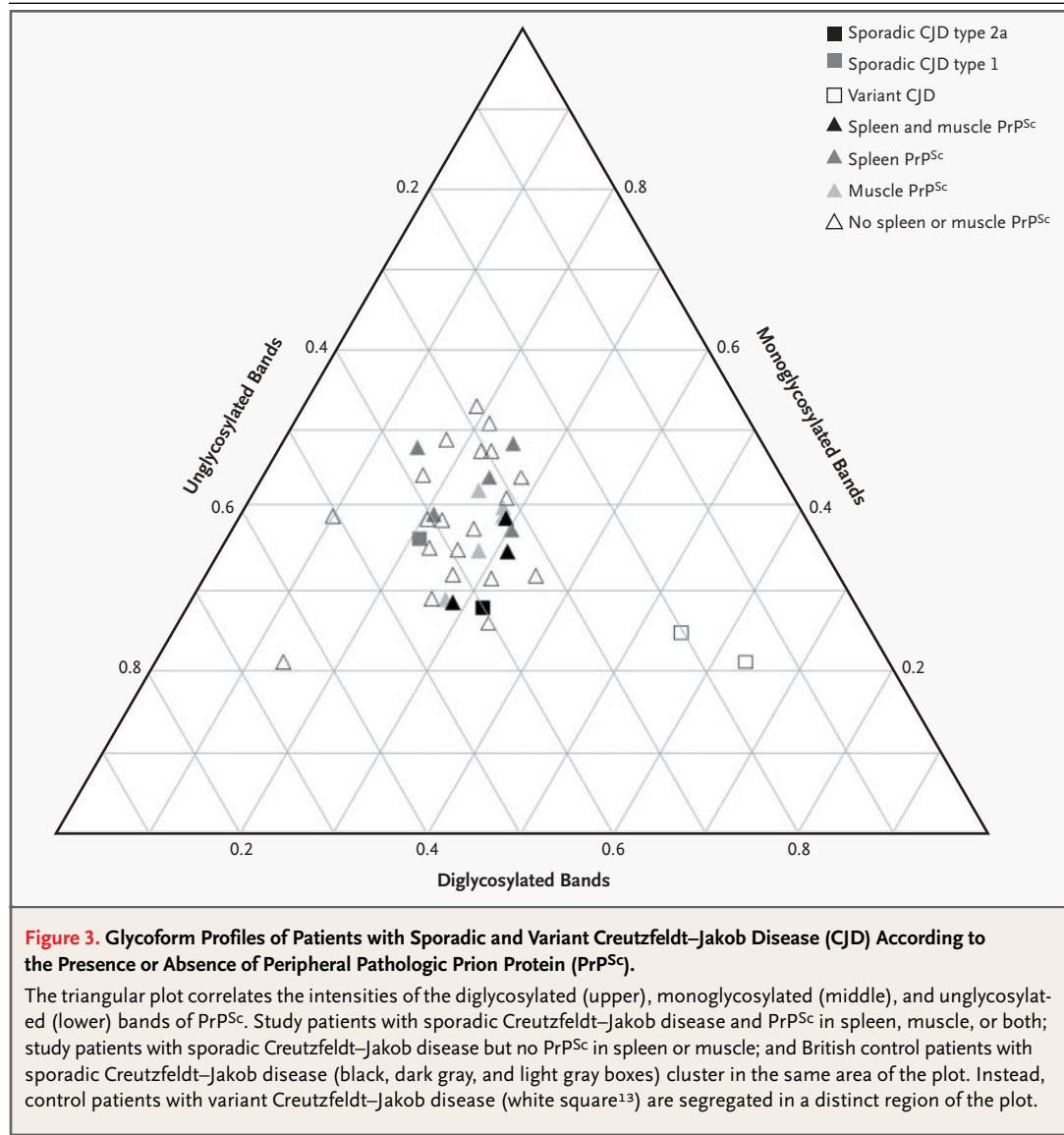
ever, after a systematic search for PrP^{Sc} in extraneural organs of patients referred to the Swiss National Reference Center for Prion Diseases, we found that extraneural deposits of PrP^{Sc} are much more frequent in patients with sporadic Creutzfeldt-Jakob disease than previously thought. There may be several reasons why this fact was not previously recognized.

First, our series of patients is much larger than previous ones. Second, repeated homogenization of distinct fragments of extraneural organs identified substantial variations in the PrP^{Sc} content of these tissues. Generic problems related to the technical reproducibility of our results are unlikely to account for this variation, since control samples consisting of brain homogenate from a patient with sporadic Creutzfeldt-Jakob disease diluted with muscle or spleen homogenate from a patient without a prion disease did not have such variations (data not shown). Instead, the presence of variability suggests that the distribution of PrP^{Sc} is not homogeneous in single muscle fibers and single splenic germinal centers. This finding parallels the finding that prion infectivity titers vary in spleen²² and muscle⁴ samples from experimentally infected mice.

Third, PrP^{Sc} levels in spleen and muscle were lower than those in brain by a factor of 1×10^{-4} : extraneural PrP^{Sc} was never detectable by plain Western blot analysis (data not shown) and could be visualized only after sodium phosphotungstic acid precipitation, an approach that increases the sensitivity of Western blot analysis by three orders of magnitude by preferentially precipitating PrP^{Sc} from tissue homogenates.^{8,11} Although this method is not suitable for use with very small biopsy specimens (less than 100 mg), it is ideal for situations in which sample size is not a limiting factor.

Previous studies suggested the presence of abnormal PrP in the skeletal muscle of patients with inclusion-body myositis.²³ However, this may represent denervation-dependent up-regulation of PrP^C transcription.²⁴ In one patient with coincident sporadic Creutzfeldt-Jakob disease and inclusion-body myositis, we found PrP^{Sc} levels that were 10^3 times as high as the levels in our study cohort, possibly because the overexpression of PrP^C in muscle facilitates the deposition of PrP^{Sc} (Kovacs G: personal communication).

Does the presence of peripheral PrP^{Sc} correlate with any specific genetic, biochemical, or clinical



factors? When plotting the incidence of codon 129 polymorphisms (MM, MV, or VV [homozygous for valine]), PrP^{Sc} core-fragment sizes (type 1, 21 kD, and type 2, 19 kD), and histologic characteristics (plaque and plaque-like lesions [P], cortical or synaptic deposits [C], or thalamic deposits [T]), we observed several uncommon phenotypes in patients with peripheral PrP^{Sc}, such as MM2C and MV2. In addition, one patient with PrP^{Sc} in lymphoid organs who was homozygous for valine at codon 129 could not be classified according to any of the existing schemes.^{18,25} These features may represent a new phenotype with atypical clinical signs,²⁶ which we have termed VV1P because of the presence of

plaque-like deposits of PrP in the cerebellum and a PrP^{Sc} core-fragment size of 21 kD.

The relative ratios of PrP^{Sc} glycoforms are different in sporadic and variant Creutzfeldt–Jakob disease. Glycotype analysis failed to reveal features specific to patients with peripheral PrP^{Sc}, and none of them had the glycoform distribution associated with variant Creutzfeldt–Jakob disease. Patients with splenic PrP^{Sc} had significantly longer durations of disease than did those without splenic PrP^{Sc}. Assuming that prions arise primarily in the central nervous system of these patients, one might speculate that protracted disease increases the likelihood of spillover of cerebral PrP^{Sc} to extraneural areas.

Although the amount of PrP^{Sc} found in spleen and muscle samples from patients with sporadic Creutzfeldt-Jakob disease was much lower than levels in lymphoid organs of patients with variant Creutzfeldt-Jakob disease,⁷ and the infectivity of prions from these tissues awaits verification, our findings arouse concern about the possibility of iatrogenic transmission of sporadic Creutzfeldt-Jakob disease. Brown et al. reported that extraneural tissues of patients with spongiform encephalopathy (including hereditary forms and kuru) occasionally transmitted disease when inoculated into non-human primates.²⁷ Infectivity was detected in 2 of 4 lung-tissue samples, 4 of 35 liver specimens, 5 of 28 kidney specimens, 3 of 31 spleen samples, and 3 of 15 lymph-node samples.²⁷ Five attempts to transmit the disease through the inoculation of skeletal muscle were not successful, possibly because the sample size was insufficient, because the infectivity of the samples was not uniform, or because transmission from human to nonhuman primates is not always highly efficient.

The causation of sporadic Creutzfeldt-Jakob disease is unknown. Some allegedly sporadic cases may in reality have an iatrogenic origin, and surgery of any kind was found to constitute a mild risk factor for sporadic Creutzfeldt-Jakob disease.²⁸ Extensive epidemiologic surveys did not substantiate any risk of blood-borne transmission,²⁹ suggesting that the presence of PrP^{Sc} in lymphoid organs whose cellular constituents migrate to and from blood may not lead to substantial contamination of blood donations by prions.

Many patients with sporadic Creutzfeldt-Jakob disease are subjected to extensive neurologic examinations in the prodromal stages, often including electromyography and muscle biopsies. The finding that PrP^{Sc} is prevalent in skeletal muscle reinforces calls for the use of single-use needle electrodes and of special protocols for the sterilization of surgical instruments used for muscle biopsies.

Our results suggest that muscle and lymph-node

biopsies can be used as diagnostic procedures for sporadic Creutzfeldt-Jakob disease. Because of the unambiguous and extremely specific nature of the immunochemical detection of PrP^{Sc}, any positive results would firmly establish the diagnosis of Creutzfeldt-Jakob disease without the need for more invasive procedures. However, the diagnostic sensitivity of the detection of PrP^{Sc} in muscle-biopsy specimens is likely to be low, since less than one third of our patients were positive for PrP^{Sc} in muscle. We did not detect PrP^{Sc} in one muscle-biopsy specimen obtained because of suspected vasculitis 10 weeks before death in a patient with Creutzfeldt-Jakob disease (data not shown). The sensitivity of prion detection is likely to improve soon, in view of the considerable refinements in the technique that are being reported.³⁰

In contrast to previous reports, our study focused on a homogeneous, narrowly defined group of patients. All the patients had died of sporadic Creutzfeldt-Jakob disease in Switzerland between 1996 and 2002. On the other hand, there has been an alarming increase in the incidence of Creutzfeldt-Jakob disease in Switzerland.¹³ Although the causes of this epidemiologic shift are not clear, the etiologic process of sporadic Creutzfeldt-Jakob disease in our cohort may differ from that in cohorts in other countries. It will therefore be important to extend the present study to additional countries, in order to determine whether our observations apply to all cases of sporadic Creutzfeldt-Jakob disease.

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