Loss of Cerebellar Granule Neurons Is Associated With Punctate but Not With Large Focal Deposits of Prion Protein in Creutzfeldt-Jakob Disease

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INTRODUCTION

Human prion diseases are fatal neurodegenerative disorders that belong to the group of transmissible spongiform encephalopathies and occur worldwide; they can be sporadic, genetic, or infectious. Most patients afflicted with these diseases develop rapidly progressive dementia and focal neurological signs such as cerebellar ataxia, extrapyramidal or pyramidal symptoms, myoclonus, and akinetic mutism (1). The most frequent human spongiform encephalopathy is sporadic Creutzfeldt-Jakob disease (sCJD), which is of unknown etiology and accounts for 80% of cases; there is an estimated yearly incidence of 1 to 1.5 per million (2).

The major neuropathologic features of these disorders are spongiform degeneration, neuronal loss, synaptocystic alterations, astrogliosis, and microglial activation. There are also accumulations of the conformationally abnormal disease-associated isoforms of the prion protein (PrP) that are misfolded and are resistant to proteinase K digestion (3). These isoforms are designated PrPres or PrPSc (Sc is in reference to the prion disease scrapie). The abnormal forms of PrPSc copurify with infectivity, aggregate to form deposits, some of which exhibit amyloid properties, and cause the characteristic neuropathologic changes in animal and human prion diseases (2). Indeed, the accumulation of altered forms of PrPSc in the brain has been proposed to be an early causative event of neurodegeneration (4). Numerous experimental studies using biochemical, cellular, molecular, and transgenic techniques have clarified the chemical and infectious properties of the infectious agent, but the cellular events associated with neurodegeneration and neuronal death remain poorly understood in humans. Most studies of the relationships between PrP accumulation and brain lesions have focused on spongiform changes in animal models and in human cases, but whether neuronal loss correlates with PrP deposition is an open question. In some experimental models, PrP could not be detected, although there were characteristic pathological alterations; whereas in other models, PrP accumulations have been seen in the absence of clinical signs or pathological findings (5, 6). Moreover, in experimental models, particularly transgenic mice, the pathology may differ from that in naturally occurring diseases. Therefore, it is important to characterize the types of PrPSc deposition associated with neuronal loss in human prion diseases.

Abstract

Whether aggregates of prion protein (PrP) reflect neurotoxicity or are neuroprotective in prion diseases is unclear. To address this question, we performed a clinicopathologic study of cerebellar granular neurons in 100 patients affected with sporadic Creutzfeldt-Jakob disease (CJD). There was significant loss of these neurons in the subset of cases with Val/Val genotype at PRNP Codon 129 and Molecular Isotype 2 of abnormal PrP (sporadic CJD-VV2) (n = 32) compared with both the other CJD subtypes and to controls. Pathological PrP deposits of the punctate-type (synaptic-type) in this subgroup correlated with neuronal loss and proliferation of astrocytes and microglia. By contrast, the numbers of large deposits (5- to 50-μm-diameter) and numbers of amyloid plaques did not correlate with neuronal loss. These findings are consistent with the view that large aggregates may protect neurons by sequestering neurotoxic PrP oligomers, whereas punctate deposits may indicate the location of neuronal death processes in CJD.

Key Words: Astrocyte, Cerebellum, Creutzfeldt-Jakob disease, Microglia, Neuronal loss, Prion protein.
There is considerable clinical and pathological heterogeneity among patients with sCJD, and distinct phenotypic variants have been described. The diversity of sCJD forms has been related to the protease-resistant core of PrP\(\text{res}\) (Isotypes 1 and 2, also called 2A, of PrP\(\text{res}\)) detected in the brain and the Met/Val polymorphism at Codon 129 of the PrP gene (PRNP). These 2 factors are associated with brain regional deposition of abnormal PrP and major histological changes (7). The aim of the present study was to obtain a better understanding of the neuropathologic processes associated with neuronal loss in CJD. We focused our attention on the atrophy of the granule cell layer of the cerebellum that is associated with ataxia. Because the loss of cerebellar granule neurons substantially varies among patients and can be quantified by accurate methods, this vulnerable neuronal population is an appropriate anatomical site for assessing relationships between the degree of neuronal loss, PrP deposition, and activation of astrocytes and microglia. In the subgroup of sCJD-VV2 patients, there were strong correlations between the numerical density of cerebellar granule neurons, astrocytes, and microglial cells determined in the same areas of adjacent sections. The severity of neuronal loss and extent of microglial and astrocytic proliferation also strongly correlated with PrP deposits of the punctate and diffuse type, but there was no association between the numbers of large focal deposits and neurodegeneration. These results support the hypothesis of a possible sequestration of highly toxic forms of PrP in some forms of large aggregates.

**MATERIALS AND METHODS**

**Patients and Control Samples**

Archival brain specimens were obtained at autopsy from 100 cases of definite sCJD with no genetic alteration in PRNP coding region and 31 control cases with no disorders involving the cerebellum from a series of clinically assessed patients. The groups did not significantly differ with respect to mean age at death, time interval from death to tissue fixation, or gender ratio. The study was conducted in accordance with French legislation. Informed consent for an autopsy was given by the patients' relatives. Patient diagnoses were confirmed by histopathology, immunohistochemistry, and biochemistry. Tissue specimens from additional cases were used as positive or negative controls for immunostaining experiments.

After fixation in 10% formaldehyde solution, the brains were dissected and samples for histology were placed into cassettes. Formalin-fixed tissue blocks were placed in 96% absolute formic acid to decrease infectivity; they were then processed routinely, embedded in paraffin, and cut in serial sections for neuropathological and immunohistochemical procedures (8). Staining with hematoxylin and eosin, periodic acid-Schiff, Congo red, and Bodian silver impregnation coupled to Luxol fast blue were used for neuropathologic examinations. Sections from all cases were examined by the same observers (Jean-Jacques Hauw and Véronique Sazdovitch) in the absence of any patient information. Accumulation of the pathological form of PrP was systematically investigated both in tissue sections after immunohistochemistry, including an autoclaving step in citrate buffer, and in tissue homogenates after preparation of scrapie-associated fibrils and separation of proteins by electrophoresis. The types of protease-resistant isoforms of PrP (PrP\(\text{res}\)) were determined by Western blotting of tissue extracts from various regions of the contralateral half of the brain that had been frozen immediately after autopsy and kept at \(-80^\circ\text{C}\) pending analyses. The PrP\(\text{res}\) isotype detected was classified Type 1 or 2 (also called 2A) on the basis of its molecular mass/glycoforms ratio and as reported by Parchi and colleagues (7, 9, 10).

**Quantification of Cellular Densities**

The numerical densities of cerebellar granule neurons, astrocytes, and microglia were estimated in adjacent 7-\(\mu\)m-thick sections of the cerebellar vermis that included cortex and dentate nucleus, and sections were cut perpendicular to the brainstem axis. Quantification was performed using a computer-assisted image analysis system consisting of a Leica (Wetzlar, Germany) Laborlux D microscope equipped with an XY-stage, a z axis micrometric sensor (Avaxe VRZ2405 module, Heidenhain, Versailles, France), an Exwaxe color video camera (Sony, Japan), a PC workstation, and Mercator software (Pro-Version v1.79c, Explora Nova, La Rochelle, France). Cerebellar granule neurons were counted in 20 fields regularly distributed in the tissue section in the middle of the granular cell layer. The positions of the counted neurons were plotted on the computer screen showing both the tissue image that was transmitted by the camera and the cartography of the studied region. Plotting locations in tissue sections were recorded for further quantification in adjacent sections of the corresponding cellular density for glial fibrillary acidic protein (GFAP)\(^+\) or CD68\(^+\) cells and for focal PrP deposition. Neuron nuclei were identified by their spherical shapes and sizes in tissue sections stained with 0.1% (wt/vol) hematoxylin and eosin. Irregularly shaped nuclei with extensive chromatin that were characteristic of glial cells were not recorded. The neuronal nuclei were counted within an unbiased counting frame when the top of the nuclei could be observed in the tissue section (11) using a \(\times\) 100 oil-immersion objective with a short depth of focus (100/1.25 numerical aperture, Leitz). The size of the counting frame (24 \(\mu\)m \(\times\) 35 \(\mu\)m; area, 840 \(\mu\)m\(^2\)) was chosen to obtain cell counts with a coefficient of error (CE) between measurements lower than 10% and was suited to the anatomical organization and neuronal density in glomeruli of the cerebellar granule cell layer. The CE was calculated from the coefficient of variation (CV) divided by \(\sqrt{n}\), where \(n\) was the number of values and coefficient of variation was the SD of the \(n\) values divided by the mean of these \(n\) values. In cases with extensive neuronal loss, measurements were performed at 25 sites to keep values of the coefficient of error low. Glial cells were counted in 1 of 2 previously examined fields in the granular layer and in 10 adjoining fields located in the bordering molecular layer. CD68\(^+\) cells were counted with an \(\times\) 40 oil-immersion objective (40/1.30 numerical aperture, Leitz). The limits of the analyzed areas were drawn on the computer screen over the images of the cerebellar layers, and a mean surface of approximately 410,000 \(\pm\) 13,000 \(\mu\)m\(^2\) was scrutinized in each layer. The GFAP\(^+\) cells were examined using an \(\times\) 100 objective (100/1.25 numerical aperture, Leitz). They were only counted if the upper surface of the nucleus...
was encountered within the counting frame. A larger counting frame (48 μm x 65 μm; area, 3,120 μm²) was chosen because the number of those cells was low. Astrocytes were plotted in 40 counting frames corresponding to the upper, lower, left, and right positions of the fields quantitated for CD68⁺ cells in the granular layer; they were also examined in 40 corresponding areas in the molecular layer. Stained tissue sections were coded and analyzed in a randomized order. The dissector volume of tissue through which the neurons and astrocytes were counted (Vdis) was calculated from the area of the counting frame (Aframe) and the measurement of z displacement in the tissue width (H, section thickness). The optical dissector volume of tissue was given by Vdis = (Aframe) (H). Neuronal and astroglial density (Nv) was calculated by Nv = Q/ Vdis, where the number of counted cells present in the reference section was Q. A similar calculation was performed for microglial cells. Within each case, neurons were sampled through approximately 0.11 x 10⁶ μm³ of granular layer, astrocytes were sampled through approximately 0.85 x 10⁶ μm³ of granular layer and the same volume of adjoining molecular layer, and microglial cells were sampled through approximately 4.10 x 10⁶ μm³ of granular and approximately 4.10 x 10⁶ μm³ of molecular layer. The numerical density of cerebellar granule neurons was not significantly correlated with postmortem interval or with the duration of histological fixation.

**Quantification of PrP Deposition**

The accumulation of immunostained PrPSc was examined in the granular and molecular layers of the cerebellum. Visual qualitative evaluation was performed and semiquantitative scores (0–4 grading scales) were assigned for global and various types of deposit staining (punctate and diffuse small granular staining, also called synaptic-type; granular, <5-μm-wide scattered nonamyloid deposits; focal, 5- to 50-μm-wide nonamyloid rounded deposits, called plaquelike deposits). For punctate deposits, a score of 0 representing totally unstained tissue and semiquantitative scores of 1, 2, 3, and 4 were assigned, indicating that staining was weak, moderate, strong, or very strong, respectively. The focal deposits 5 to 50 μm in size were counted in the molecular layer where PrP staining is in strong contrast to surrounding tissue using computer-assisted image analysis. The sampling areas were precisely adjusted to those examined for CD68⁺ cells counting in adjacent sections. The number of focal PrP deposits per mm² of tissue section was calculated.

**Antibodies**

Mouse monoclonal antibodies (mAbs) were used to detect human PrP (12F10 clone recognizing the 142–160 amino acid sequence, 1:200 dilution; SpiBio, Massy, France; 3F4 clone recognizing the 109-112 sequence, 1:1000 dilution; SpiBio), human GFAP (6F2 clone, 1:500 dilution; DakoCytomation, Glostrup, Denmark), human β chain of HLA-DR, -DQ, -DP antigen (CR3/43 clone, 1:50 dilution; DakoCytomation), and human activated macrophage/microglia CD68 (KP1 clone, 1:1000 dilution; DakoCytomation). A semiquantitative study of HLA-DR⁺ and CD68⁺-immunoreactive cells was performed to validate the data on CD68⁺ microglial activation and showed a strong positive correlation between the 2 markers for both the staining and numbers of positive cells.

**Immunohistochemistry**

Serial sections of formalin-fixed specimens were mounted on SuperFrost glass slides (Milian, Bron, France) and incubated with specific antibodies after relevant pretreatments. An automated method (Ventana Medical Systems SA, Illkirch, France) was used to obtain optimum reproducibility of the staining. The iVIEW DAB detection kit and the Ventana NexES automation were used at 37°C as described (12). To detect PrPSc deposits, antigens were retrieved by hydrated autoclaving (100°C, 25 minutes) and 99% formic acid (room temperature, 3 minutes) before the automated procedure. This optimized method yields a consistent labeling of PrPSc in the brain of patients with prion diseases, with no revelation of cellular isoforms of PrP in control cases (12). Similarly, pretreatment by microwave (400 W) and boiling in sodium citrate buffer (10 mmol/L, pH 6.0, 20 minutes) was performed for CD68 detection.

**Western Blot Analysis**

Frozen cerebellar tissue (350–370 mg) was used to prepare scrapie-associated fibrils from 20% wt/vol tissue homogenates in a 5% glucose solution as described by Lasmezas et al (5) with slight modifications. The clear lysate was treated with proteinase K (10 μg/mL, 37°C, 45 minutes; Merck, Darmstadt, Germany). Samples were concentrated at 27,500 × g on a 10% sucrose gradient (22°C, 2 hours). The pellet was resuspended in a denaturing buffer, and the proteins were separated by acrylamide gel electrophoresis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis 12% gel). Proteins were transferred to nitrocellulose membranes and incubated with 3F4 mAb directed against amino acids 109 to 112 of PrP (1:50,000 dilution). The standard conditions correspond to the load of samples equivalent to 4 mg of brain. After extensive washing, the signal was detected by horseradish peroxidase–conjugated secondary antibody (Immunopure goat anti-mouse IgG (H+L), 1:5000 dilution; Pierce Biotechnology, Rockford, IL), the enhanced chemiluminescence system (ECL Western blotting detection

**TABLE. Numerical Density of Cerebellar Granule Neurons**

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Neurons/mm² ± SEM</th>
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<tbody>
<tr>
<td>Control (n = 31)</td>
<td>2.7 ± 0.1 x 10⁶</td>
</tr>
<tr>
<td>sCJD-MM1 (n = 31)</td>
<td>2.6 ± 0.1 x 10⁶</td>
</tr>
<tr>
<td>sCJD-MM2 (n = 4)</td>
<td>2.8 ± 0.2 x 10⁶</td>
</tr>
<tr>
<td>sCJD-MV1 (n = 16)</td>
<td>2.6 ± 0.2 x 10⁶</td>
</tr>
<tr>
<td>sCJD-MV2 (n = 15)</td>
<td>2.7 ± 0.1 x 10⁶</td>
</tr>
<tr>
<td>sCJD-VV1 (n = 2)</td>
<td>2.8 ± 0.5 x 10⁶</td>
</tr>
<tr>
<td>sCJD-VV2 (n = 32)</td>
<td>1.8 ± 0.1 x 10⁶*</td>
</tr>
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Mean number of neurons per cubic millimeter (±SEM) within the granular cell layer of the cerebellum. Cases in the sporadic Creutzfeldt-Jakob disease (sCJD)-VV2 variant group had a significant decrease in density of these neurons compared with cases in the control group and sCJD cases characterized by a Met/Met or Met/Val genotype at Codon 129 of PrP and a Biochemical Type 1 or Type 2 of PrPSc detected in contralateral cerebellar tissue (*, p < 0.001, 1-way analysis of variance and pairwise multiple comparison procedure by Holm-Sidak method).
reagents; Amersham Biosciences AB, Uppsala, Sweden) and exposure to x-ray film (Kodak). The PrP\textsuperscript{sc} isotypes were classified in Type 1 or Type 2 (also called 2A) according to the pattern of electrophoretic migration (9, 10).

**Paraffin-Embedded Tissue Blots**

To detect the accumulation of the abnormal and disease-associated form of the PrP, paraffin-embedded 7-μm-thick serial sections were transferred onto nitrocellulose membranes and processed for immunohistochemistry (primary antibody, 3F4 clone; 1:1000 dilution; SpiBio) following a limited proteolytic digestion of PrP\textsuperscript{Sc} as described (13). Control cases showed no evidence of PrP\textsuperscript{Sc} in tissue. These paraffin-embedded tissue (PET) blots were examined using an Olympus SZX12 zoom stereomicroscope.

**Molecular Genetics**

Genomic DNA was extracted from peripheral blood leukocytes collected during life or from frozen brain tissue after death; PrP gene (PRNP) coding sequence analysis was performed on the entire open reading frame (14).

**Statistical Analyses**

Data are presented as the mean ± SEM. For statistical comparisons between groups, parametric and nonparametric tests were used according to normality and variance of data.
distribution (SigmaStat statistical program, version 3.5, Systat Software, Inc., Point Richmond, CA). The null hypothesis was rejected for α risk equal to 5%.

RESULTS

Neuronal Loss in the Cerebellar Granule Cell Layer and Clinical Data

We analyzed neuron densities in the granule cell layer of the cerebellum at 20 to 25 different sites in each section to obtain a CE between measurements lower than 10% per tissue section. In total, 41,586 cerebellar granule neurons were counted, with a CE of 0.061 ± 0.002 (mean ± SEM). The sCJD cases (n = 100) were grouped according to the genotype at PRNP Codon 129 and the biochemical type of PrPres in the cerebellum. The mean numerical density of cerebellar granule neurons was decreased in the sCJD-VV2 group compared with that in control subjects and to that in the MM1, MM2, MV1, and MV2 molecular subtypes of sCJD patients (p < 0.001; Table).

The sCJD-VV2 subgroup of 32 cases had values of numerical density ranging from levels of the control cases to levels that were decreased by as much as approximately 70% to 85% (i.e. divided by approximately 4- to 5-fold). These cases had cerebellar ataxia more often than patients of the other sCJD molecular subtypes (p < 0.001). Within the sCJD-VV2 subgroup, the numerical density of cerebellar granule neurons was lower in patients who had shown ataxia at the onset of the symptomatic phase of the neurological illness (1.5 [±0.1] × 10⁶ vs 2.2 [±0.2] × 10⁶ neurons/μL, p < 0.02); this indicates that the quantified terminal neuronal loss corresponded to initial as well as terminal symptoms. The durations of clinical illness were not correlated with neuronal loss, and neuronal counts were not significantly correlated with age, postmortem delay, or duration of histological fixation.

Cerebellar Granule Neuron Density Correlates With Astroglial and Microglial Cell Densities

The sCJD-VV2 subgroup was used to study the association between neuronal loss and glial proliferation. The numerical density of GFAP+ astrocytes and CD68/KP1+ microglial cells was investigated in tissue sections adjacent to those used to estimate neuronal density (Fig. 1). A total of 1,044 GFAP+ cells (CE, 0.165 ± 0.043 in granular layer) and 20,881 CD68+ cells were counted (CE, 0.073 ± 0.004 in...
The PrP Sc deposition was highest in patients with the most severe neuronal damage (Fig. 2). Cerebellar granule neuron degeneration in Val/Val genotype at PRNP Codon 129 and Molecular Isotype 2 of abnormal prior protein ([PrP] sporadic Creutzfeldt-Jakob disease-VV2) patients correlates with glial activation and associated with diffuse punctate PrP deposits. The relationships between mean numerical density of cells and quantitation of punctate PrP deposits (synaptic-type) (SD) are shown. Mean cell counts for neurons, astrocytes, and microglial cells were determined for each score of punctate PrP Sc deposits in the cerebellar granular layer (gL) and molecular layer (mL); values are plotted with respect to punctate PrP deposition scores. Marked loss of cerebellar granule neurons and increased glial cell densities are associated with punctate deposits detected in the gL and in the mL at some distance from the cell bodies of the cerebellar granule neurons. Cerebellar granule neurons (.), astrocytes (■, AgL; □, AmL), microglial cells (▲, MgL; △, MmL), and punctate PrP deposition in the gL (A) and mL (B).

### Decreased Cerebellar Granule Neuron and Increased Microglial and Astroglial Cell Numerical Density Correlate With Global PrP<sup>Sc</sup> Accumulation

To assess whether neuronal loss correlated with PrP deposition, 2 complementary methods for PrP detection (i.e. classical immunohistochemistry of tissue sections [12F10 mAb] and PET blot of tissue sections fixed on a nitrocellulose membrane treated with protease K [3F4 mAb]) were used. The semiquantitative scores for immunostaining levels obtained with these methods were positively correlated (granular layer: $r_s = +0.49$, $p < 0.01$; molecular layer: $r_s = +0.60$, $p < 0.001$). The PrP<sup>Sc</sup> deposition was highest in patients with the most severe neuronal damage (Fig. 2). Cerebellar granule neuron counts correlated with PrP<sup>Sc</sup> staining levels scored in the granular and molecular layers (PrP immunohistochemistry: $r_s = -0.56$, $p < 0.002$, and $r_s = -0.50$, $p < 0.01$; PrP-PET blot: $r_s = -0.42$, $p < 0.05$, and $r_s = -0.50$, $p < 0.01$, respectively, for each layer). The amount of PrP<sup>Sc</sup> deposition was also positively correlated with counts of activated microglia (PrP immunohistochemistry, granular and molecular layers: $r_s = +0.69$, $p < 0.001$, and $r_s = +0.76$, $p < 0.001$; PrP-PET blot, granular and molecular layers: $r_s = +0.47$, $p < 0.01$, and $r_s = +0.46$, $p < 0.01$). A weaker statistical correlation was observed between astrocyte counts and PrP<sup>Sc</sup> semiquantitative scores (PrP-PET blots staining levels, granular layer: $r_s = +0.37$, $p < 0.05$).

### Numerical Density of Cerebellar Granule Neurons Correlates With Punctate but Not Focal PrP<sup>Sc</sup> Deposits

Semiquantitative scores of diffuse punctate deposits (synaptic-type) in the granular and molecular layers were strongly correlated ($r_s = +0.63$, $p < 0.001$), but this was not observed for aggregated deposits (granular, <5 μm wide; focal, 5–50 μm wide). The mean cell counts for each score value of punctate PrP<sup>Sc</sup> deposits were calculated. There was a strong relationship between cell counts and accumulation of punctate PrP<sup>Sc</sup> deposits in both the granular and molecular layers (Fig. 3); the numbers of cerebellar granule neurons regularly decreased along with a corresponding increase in astrocytes. For microglial cell numbers, there was a steady increase from the lowest to the highest PrP<sup>Sc</sup> load. The strength of statistical association between punctate scores and cell counts was highest for microglial cells in the granular layer (microglia, $r_s = +0.55$, $p < 0.002$; neurons, $r_s = -0.48$,..
p < 0.01; astrocytes, $r_s = +0.41$, $p < 0.05$) and molecular layer (microglia, $r_s = +0.76$, $p < 0.001$; neurons, $r_s = -0.53$, $p < 0.005$; astrocytes, $r_s = -0.06$, not significant [NS]). By contrast, the scores for focal deposits in both sites were not associated with neuronal loss. Furthermore, focal deposits were seen in association with low as well as high loads of punctate (synaptic-type) deposits (Fig. 4A). Cases with amyloid plaques had no prominent neurodegeneration.

Focal 5- to 50-μm-wide PrP deposits were quantified in tissue sections after adjustment of areas to those measured for cell counting. The number of these large deposits per square millimeter of tissue section in the molecular layer was not correlated to cerebellar granule neuron counts (Fig. 4B). To analyze the relationship between neuronal loss and the type of PrPSc deposits, the cases were classified according to their number of focal PrPSc deposits (0–9.9, black bars; 10–19.9, red bars; 20–29.9, green bars; >30, yellow bars) and scores of punctate deposits in the gl (low, 0–3 arbitrary units, $n = 16$; high, >3 arbitrary units, $n = 16$). There is a significant association with punctate deposits ($p < 0.01$) but no association with 5- to 50-μm large focal PrPSc deposits (1-way analysis of variance, not significant). The graph shows a trend for a lower neuronal loss when amounts of large aggregates are higher for both low and high levels of punctate deposits. (E) Relationship similar to (D) but less regular between numerical density of neurons and focal PrPSc deposits when cases are classified according to scores of punctate PrPSc deposits in the ml (low, 0–3 arbitrary units, $n = 13$; high, >3 arbitrary units, $n = 19$). There is a significant association with punctate deposits ($p < 0.05$) but no association with 5- to 50-μm large focal PrPSc deposits (1-way analysis of variance).
lower when amounts of 5- to 50-μm deposits were higher (Fig. 4C). There was also no relationship between the number of focal PrPSc deposits per square millimeter and the numerical density of microglial cells located in the molecular layer (r = −0.29, NS).

**Investigation of 2 sCJD-VV1 Variant Cases**

To explore whether the results observed in sCJD-VV2 cases were caused by the patients’ Val/Val genotype at Codon 129 of PRNP and/or to PrPres Molecular Isotype 2, sections were studied in 2 cases of the rare sCJD-VV1 variant with PrPres Molecular Isotype 1 in the cerebellum. The sections were examined together with those of sCJD-VV2 cases for all series of staining and quantification. These cases were distinct both clinically and pathologically from the sCJD-VV2 cases. The patients had alterations of higher functions rather than ataxia and had 1) no loss of cerebellar granule neurons (2.7 × 10⁶ and 2.9 × 10⁶ vs 1.8 (±0.1) × 10⁶ neurons/mm²), 2) no astroglial and microglial proliferation, 3) very limited PrPSc deposition in a few small foci of punctate deposits, and 4) no focal deposits 5 to 50 μm in size (0 ± 0 deposits/mm² in each of the 2 cases). Hence, different changes observed in patients with the same genotype but different PrP isotypes indicate the role of PrPres Type 2 in the sCJD-VV2 variant.

**DISCUSSION**

Our results show strong statistical relationships between PrPres deposition, neuronal loss, and glial proliferation in the human brain. The estimation of numerical density of cells in definite anatomical locations was performed in a large sample of sCJD cases. We find that 1) the loss of cerebellar granule neurons is statistically significant in only 1 subtype of sCJD (sCJD-VV2); 2) the severity of neuronal loss is associated with the degree of PrPres deposition in the cerebellar cortex; 3) the numerical density of cerebellar granule neurons, astrocytes, and activated macrophages/microglia are strongly correlated; and 4) neuronal loss and glial proliferation are associated with PrPres deposits of the punctate (synaptic) type but not with aggregated PrPres deposits of the focal type.

**Cerebellar Granule Neuron Reduction**

Loss of neurons in the granule layer of the cerebellum has been known since 1965 when the frequent occurrence of cerebellar signs was described in small numbers of sCJD patients showing cerebellar symptoms (15–22) and subsequently in large series of cases categorizing the various forms of sCJD (7, 23). We found cerebellar neuronal loss in the sCJD-VV2 but not the other CJD variants (Table). There was, however, a wide variation within each of the subgroups. Moreover, although quantification was performed at a stage of the disease when ataxia was observed in all sCJD-VV2 patients, the mean value of neuronal density measured at postmortem was significantly lower in those cases for which a cerebellar ataxia had been diagnosed at the onset of the clinical phase (15). This result emphasizes the contribution of this neuronal loss to cerebellar ataxia and further documents and strengthens the association between ataxia and granule neuron vulnerability in the sCJD-VV2 variant, which seems to be associated with a particular isotype of PrP. The results also underscore the role of host-encoded PrP (polymorphism of PRNP at Codon 129) in combination with the molecular isotype of PrPres detected in the tissue (7).

**Astroglial Proliferation**

The proliferation of astrocytes in the cerebellum is well documented in sCJD and experimental models of prion diseases (20, 22, 24–28). Intracytoplasmic accumulation of disease-specific PrP depends on the inoculated strains probably because of variations in cell tropism and PrP processing (29–31). We found no astrocytosis in sCJD-VV patients with PrPres conformers of the biochemical isotype 1 in the cerebellum probably because of a lack of such properties. Astrocyte proliferation and PrPres deposition were significantly correlated in sCJD-VV2 cases, in agreement with experiments showing that these cells are a site of agent replication and (32–34) and possibly activated by accumulation of misfolded PrP. An increased expression of several cytokines by astrocytes might contribute to neuronal dysfunction through proinflammatory and neurotoxic pathways (35–38). Although the data reported here show that PrPres accumulation and astrocyte proliferation are correlated, the strength of their statistical association was much lower than between PrPres deposition and microglial proliferation.

**Microglial Activation**

We have shown that PrPres conformers of the biochemical isotype 2 of PrPres accumulate in the cerebellum along with microglial proliferation. Extensive proliferation of activated macrophages/microglia in cases with a high degree of neuronal loss is supported by the dual investigation of major histocompatibility complex Class II and CD68, which provided similar numbers and staining levels of positive cells. Despite the fact that microglia/macrophage activation is increased in CJD and animal models, considerable variations are reported (39–42). The present study further demonstrates that the extent of microglial proliferation is correlated with the degree of neuronal loss in sCJD-VV2 patients. The concept that neuronal loss and PrP deposition are related to macrophage/microglia activation, with microglial activation occurring before neuronal loss in association with PrPres deposition (43–47), is consistent with our findings.

Microglial cells may act as primary cytotoxic sources or be secondarily activated in response to the neurodegenerative process (46). In vitro studies have shown that the presence of microglia in coculture greatly increases neuronal death (48, 49). The toxicity of activated microglia is probably caused in part by the release of cytokines and substances involved in oxidative stress (27, 45, 48, 50). Activated macrophage/microglia may also inactivate the effects of prions at some stages of the pathological process. For example, microglial cells in the cerebellum of sCJD cases express EAAT-1 and may partially protect cerebellar granule neurons before their extensive degeneration (51). The role of microglia in the pathophysiological process leading to neuronal death remains largely unknown, however, especially at critical stages in the course of prion diseases.
PrP Deposition

The relationship between the accumulation of PrPSc and neurodegeneration might reflect the neurotoxicity of abnormal PrP conformers. Their role in prion diseases has been observed in natural diseases and investigated in vitro and in vivo but remains poorly understood. The PrPSc may induce neuronal damage both indirectly through microglia and astroglia and directly through pathological effects of trans-conformed host PrP within vulnerable neurons (4, 52, 53). The present results show that the strongest statistical association between PrP deposition and numerical density of cells in human cases corresponds to microglial proliferation, which is also the first variable to increase when PrP accumulates as punctate deposits. The observation that punctate deposits are related to the severity of neuronal loss is compatible with the impairment of synapses located in glomeruli of the granular layer and between parallel fibers and Purkinje cell dendrites in the molecular layer where neuronal death may occur after synaptic and neuronal injury (54). Because experiments in mice with gene invalidation focused on targeted cell types support possible roles for intraneuronal and extracellular astrocytic accumulation of abnormal PrP, distinct mechanisms, possibly with reciprocal potentiation, may be involved in the neurodegenerative process of experimental (33, 55) and human CJD.

High Densities of Large Focal PrPSc Deposits Are Not Associated With Neuronal Loss

We found that neuronal loss was low when the number of focal deposits was high, making it unlikely that PrPSc conformers aggregated in large deposits could be directly associated with neurodegeneration. Indeed, the numbers of neurons in the granular layer were strongly associated with the levels of punctate PrPSc deposits both in the granular and molecular layers. In contrast, whatever the amount of punctate deposits, the neuronal loss did not increase with higher numbers of large focal deposits. Similarly, microglial proliferation was correlated with punctuate deposits and not with the density of large aggregates. Taken together, these findings are in accordance with the emerging view that some aggregated deposits might sequester neurotoxic PrPSc and impede its toxicity and/or infectivity in prion diseases. Although highly infectious and/or pathogenic isolates of PrPSc are often fibrillar, the amyloid formation is suspected to be a protective mechanism (52, 56, 57). A study of the hippocampus of prion-infected mice has shown that most tissue-bound PrPSc was located in small neurites of the neuropil and remained protease sensitive, with no amyloid deposits (58). This indicates that soluble oligomeric forms of PrPSc may be harmful in rodent scrapie infection. Our observations suggest that this may also apply to the human sCJD-VV2 variant.

In conclusion, we have shown that neuronal loss is strongly correlated with glial proliferation and accumulation of extracellular pathological PrP in punctate deposits (synaptic type) in the cerebellum of sCJD-VV2 patients. We found that there was a lack of relationship between the accumulation of 5- to 50-μm large PrP deposits and neurodegeneration, which is in accordance with the assumption that small subfibrillar particles may be more neurotoxic than large amyloid plaques in the human brain (59). Further studies are needed to elucidate the structural and biologic properties of PrP deposits. It will be crucial to understand their contribution to the pathological process in human prion diseases to develop effective neuroprotective treatments aimed at blocking the cytotoxic effects of PrP.

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