Similar genetic susceptibility in iatrogenic and sporadic Creutzfeldt-Jakob disease

Jean-Philippe Deslys,* Dominique Marcé and Dominique Dormont

Laboratoire de Neuropathologie Expérimentale et Neurovirologie, CRSSA/DSV/DPTE Commissariat à l’Energie Atomique, B.P. 6, 92265 Fontenay-aux-Roses, France

Creutzfeldt-Jakob disease (CJD) is one of the transmissible spongiform encephalopathies (TSEs). In all TSEs host susceptibility is an important factor in the development of clinical disease. The prion protein (PrP) gene appears to confer the main component of this susceptibility. The appearance of spontaneous neurodegeneration in PrP transgenic mice carrying a human mutation has raised the possibility that the origin of sporadic CJD is solely genetic. We studied PrP codon 129 polymorphism in 23 of the 25 CJD cases in France related to human growth hormone (hGH) therapy. They constitute the largest and most homogeneous hGH-related iatrogenic CJD population yet analysed. All these CJD cases were homozygous at codon 129, compared with only 50% in the healthy control group (*P < 0.00002). These iatrogenic cases also displayed a genotype frequency distribution similar to that observed in sporadic CJD. These results underline the importance of the PrP gene and especially the homozygous codon 129 genotype in determining the risk of developing CJD after contamination by a TSE agent. They also suggest that highly susceptible individuals may exist and raise the possibility that sporadic CJD may have an environmental origin.

Introduction

The unusual resistance to sterilization procedures of the causal agents of transmissible spongiform encephalopathies (TSE), also designated unconventional viruses or prions, has unfortunately been illustrated by the occurrence of Creutzfeldt-Jakob disease (CJD) in children and young adults after treatment with human growth hormone (hGH) extracted from large numbers of pooled cadaveric hypophyses. However, the peripheral route of administration used which is known to be poorly efficient in transmitting the disease, raises the question of the importance of host susceptibility in the development of CJD. This susceptibility is governed by the prion protein (PrP) gene (Prusiner, 1991; Brown, 1992; Chesebro, 1992), whose central role was recently confirmed by experiments with transgenic mice. Mutations at codons 102, 117, 178, 198 and 200 have been reported in human familial forms of TSE (Hsiao et al., 1989, 1992; Doh-Ura et al., 1989; Goldfarb et al., 1991; Goldgaber et al., 1989). Some lines of transgenic mice with a mutated PrP gene (equivalent to the human codon 102 mutation) spontaneously develop spongiform encephalopathy (Hsiao et al., 1990). However, in humans, none of these mutations are associated either with sporadic CJD, which constitutes about 90% of cases, or with iatrogenic CJD.

A significant difference between CJD cases and normal controls has been reported at the polymorphic amino acid residue 129 of PrP, where an ATG to GTG change results in a methionine to valine (Met to Val) substitution (Goldfarb et al., 1989). Forty of a series of 45 sporadic CJD cases (89%) were found to be homozygous (Met/Met or Val/Val) at codon 129 (Palmer et al., 1991) whereas in another series of 106 controls, homozygosity was found in only 49% (Owen et al., 1990). In addition, four out of seven pituitary hormone-related CJD cases in the U.K. were homozygous for valine (57%), one was homozygous for methionine (14%) and two were heterozygous (Met/Val; 29%). Contrastingly 24% of the sporadic CJD cases and only 12% of the controls were found to be homozygous for Val-129 (Collinge et al., 1991). This excess of the Val/Val genotype in affected children seems to indicate that this genotype is associated with a genetic predisposition to CJD after exposure to TSE agents in the environment. The absence of this excess of Val/Val genotype in sporadic CJD has been suggested to reflect a difference of origin: the sporadic cases might not have an environmental cause but might result from the spontaneous conversion of normal PrP (PrP0) into a pathological protease-resistant isofrom (PrP(rCJD)) responsible for the disease (Palmer et al., 1991). This interpretation is consistent with the appearance of spontaneous neurodegeneration in transgenic mice, and supports the ‘genetic only’ hypothesis linking the origin of sporadic CJD to somatic mutations.
To investigate individual susceptibility to iatrogenic CJD we studied the polymorphism of codon 129 of the PrP gene (PRNP) in 23 of 25 growth hormone-related CJD cases detected in France. These cases belonged to the group of 1698 children treated with extracted hGH before 1985, including 800 patients potentially exposed to contaminated hGH (Job et al., 1992; Clément et al., 1992).

Methods

Detection of PrP. PrPse was purified according to previously published methods for purifying scrapie-associated fibrils (SAF) (Diringer et al., 1983; Kascak et al., 1985; Hope et al., 1986). The equivalent of 0.5 mg of brain was loaded onto wells of a 15% SDS-polyacrylamide gel. For the protease resistance test, samples were subjected to digestion by 10 μg/ml proteinase K for 1 h at 37 °C and then denatured in SDS-PAGE sample buffer. Electrophoresis, electrophoretic transfer and Western blot detection by enhanced chemiluminescence were performed according to standard procedures. For immunodetection, we used an anti-PrP s° hamster monoclonal antibody kindly provided by Dr R. J. Kascak (Institute for Basic Research, Staten Island, N.Y., U.S.A.) As PrPse does not copurify with SAF, no reaction is seen in the negative control.

PRNP amplification. Genomic DNA was extracted using standard techniques. The PRNP gene was amplified by PCR between bases 142 and 819 (including the coding region between codon 48 and the end of the open reading frame). The primers used were 5’ CGCTACCCACCTCAGGGCGG Y (sense) and 5’CCGCCTCCCTCAAGCTGGAA Y (antisense). Samples of 50 μl, each containing standard buffer (1.5 mM-MgCl2), 200 μM of each dNTP and 250 nM of each primer, 100 ng of DNA, and 1 unit of Taq polymerase (Boehringer) were subjected to 35 cycles of amplification (94 °C for 30 s, 59 °C for 1 min and 72 °C for 1 min).

Allele-specific oligonucleotide hybridization. Samples (10 μl) were run on a 1% agarose gel and visualized by ethidium bromide staining. After Southern blotting under vacuum in alkaline denaturing conditions, nylon filters (Hybond-N, Amersham) were fixed by exposure to u.v. for 5 min. Filters were prehybridized for 1 h at 37 °C in 5 x SSPE (1 x SSPE is 0.15 M-NaCl, 10 mM-sodium phosphate pH 7.4 and 1 mM-EDTA). 5 x Denhardt’s solution, 0.5% SDS and 100 μg/ml denatured salmon sperm DNA. Allele-specific oligonucleotides (Met-129: 5’ CGGCTACATGCTGGG 3’, Val-129: 5’ CGGCTACGTGCTGGGG 3’, Val-117 PvuII+: 5’ CTGCAGCGGCTGGGG 3’, and Ala-117 PvuII+: 5’ CTGCAGCAGCTGGGA 3’) were radiolabelled by T4 kinase (solution comprising 25 pmol-oligonucleotide, 8 pmol [γ-32P]ATP 185 TBq/nmol, and 5 units of T4 kinase in 20 μl). Hybridization was performed for 2 h under the same conditions with 106 c.p.m. per ml 32P-labelled oligonucleotide. Filters were washed twice for 5 min at room temperature in 2 x SSPE/0.1% SDS and once for 15 min at a high stringency temperature (52 °C for Met-129, 54 °C for Val-129, 55 °C for Ala-117 PvuII+ and 57 °C for Ala-117 PvuII–) with a final 5 min wash at room temperature. Autoradiography was then performed for 2 h.

Results

PrPc\textsubscript{CJD} accumulation

In eight of the 23 children with hGH-related CJD (i.e. the iatrogenic group) the diagnosis had already been neuro-pathologically confirmed (Billette de Villemeur et al., 1992; Job et al., 1992). PrPc\textsubscript{CJD} accumulation was demonstrated in the four brain samples we analysed (Fig. 1). This abnormal PrP exhibited its two usual in vitro properties: aggregation in the presence of detergent and partial resistance to degradation by protease.

Polymorphism of PRNP codon 129

After DNA extraction from either blood or brain samples, PCR amplification and Southern blotting were performed prior to PrP codon 129 genotype analysis by allele-specific oligonucleotide hybridization. Our results showed that of the 23 children with iatrogenic CJD tested all were homozygous at codon 129 (Val/Val: 43%, Met/Met: 57%, Fig. 2 and Table 1). In addition, two parallel series were run, comprising respectively 69 healthy controls and 138 healthy children treated with hGH. The genotype frequencies of these two groups were respectively Met/Val: 54%, Val/Val: 10% and Met/Met: 36% and Met/Val: 44%, Val/Val: 12% and Met/Met: 44% (Table 1). In the group with iatrogenic CJD, excess homozygosity was statistically significant compared with the control ($\chi^2 = 18.46$, $P < 0.0002$; with correction for continuity). In our normal control group, the distribution of genotype frequencies was comparable to the one published by Collinge’s group (Owen et al., 1990; 51%/12%/37%; $\chi^2 = 0.22$, $P = 0.89$). In our iatrogenic CJD group, the 100% homozygosity was not statistically different from the value reported for sporadic CJD (Palmer et al., 1991; 89% homozygosity; $\chi^2 = 1.37$, $P > 0.24$).

Regarding the proportions of Val/Val to Met/Met genotypes, there was no significant difference between our iatrogenic CJD group (10/13) and our control group (7/25; $\chi^2 = 2$, $P > 0.15$) or the sporadic CJD group.
Susceptibility to Creutzfeldt-Jakob disease

Fig. 2. Codon 129 genotypes. Lanes 1 to 15: samples from children with iatrogenic CJD; lane 16: PCR-negative control; lanes 17 and 18: parents of child no. 2 (heterozygous control). Ethidium bromide staining shows the fragment amplified by PCR. Autoradiograms after allele-specific oligonucleotide hybridization (Met-129 and Val-129). All the children are homozygous.

Table 1. Polymorphism of PrP codon 129

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Met/Val</th>
<th>Val/Val</th>
<th>Met/Met</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>54% (37)</td>
<td>10% (7)</td>
<td>36% (25)</td>
<td>100% (69)</td>
</tr>
<tr>
<td>Controls given hGH</td>
<td>44% (61)</td>
<td>12% (16)</td>
<td>44% (61)</td>
<td>100% (138)</td>
</tr>
<tr>
<td>CJD patients given hGH</td>
<td>0% (0)</td>
<td>43% (10)</td>
<td>57% (13)</td>
<td>100% (23)</td>
</tr>
</tbody>
</table>

Fig. 3. PvuII non-coding polymorphism at codon 117. Lanes 1 to 18: identical to the corresponding lanes in Fig. 2. Children 5 and 8 are heterozygous.

Polymorphism of PRNP codon 117

As a control, we also studied the natural polymorphism at codon 117 of the PrP gene: a GCA to GCG substitution eliminates a PvuII restriction site without changing the amino acid encoded (Ala) (Doh-Ura et al., 1989). Two of the 23 children with iatrogenic CJD (9%), Fig. 3) exhibited this substitution: this percentage is described by Palmer et al. (1991) (11/29; $\chi^2 = 1.04$, $P > 0.30$). Consequently, there was no significant difference between the distribution of genotype frequencies in our iatrogenic CJD group and that observed in the above sporadic CJD group (11% / 24%; 65%; $\chi^2 = 4.50$, $P > 0.10$). The genotype frequencies observed in our control hGH-treated group were not statistically different from those of our normal controls ($\chi^2 = 1.65$, $P > 0.43$).
similar to that found in both the normal controls (7/69 i.e. 10%) and the hGH-treated controls (13/138 i.e. 9%).

Search for PRNP mutations

We also looked for the presence of the previously published mutations of the PrP gene (in codons 102, 117, 178, 198 and 200), but none of the 23 hGH-treated children with CJD exhibited any of these mutations.

Discussion

We have shown that all the 23 children with iatrogenic CJD tested out of the 25 French cases contaminated by extracted hGH were homozygous for PrP codon 129, thus demonstrating the existence of a strong link between homozygosity at this codon and the development of CJD after iatrogenic contamination. In addition, the distribution of the Val/Val and Met/Met genotypes (43/57%) suggests that this represents a true gene polymorphism, not merely a rare substitution and that homozygosity constitutes a factor predisposing to the development of CJD regardless of the amino acid encoded.

However, although all our patients were homozygous, the existence of 50% homozygous individuals in the normal Caucasian population (Owen et al., 1990) and the observation of heterozygous individuals in previously reported cases of both iatrogenic and sporadic CJD (Doh-Ura et al., 1989; Goldfarb et al., 1989; Collinge et al., 1991; Palmer et al., 1991), indicate that analysis of codon 129 alone cannot constitute a reliable predictive indicator. Furthermore, the existence of 50% heterozygotes in normal Caucasian populations (our normal controls and those of Owen et al., 1990) is not an absolute rule for all populations; for instance, only 8% heterozygotes have been reported in the normal Japanese population (Doh-Ura et al., 1991). Taking these data into account, we concluded that the present investigation required the inclusion of a second control group consisting of patients suffering from growth hormone deficiency. Consequently, 138 children treated with hGH were tested, and the results observed in this second control group were not statistically different from those obtained in the normal controls.

Our results for hGH-linked CJD are similar to those observed for sporadic CJD (Palmer et al., 1991): as codon 129 constitutes the sole known marker of genetic susceptibility to CJD in non-familial CJD, the genetic pattern of this susceptibility seems to be identical in iatrogenic and sporadic CJD. Having studied a larger more homogeneous group of iatrogenic CJD patients (in France, contamination of hGH probably occurred within a limited period of time, between January 1984 and June 1985; Job et al., 1992; Clément et al., 1992), we did not find the previously reported excess of the Val allele which supported the hypothesis that the origin of sporadic CJD was not environmental (Collinge et al., 1991; Palmer et al., 1991).

All the children treated during the same above period with hGH batches extracted from pools of large numbers of cadaveric hypophyses (including our 23 iatrogenic CJD cases and the 138 healthy controls), were subject to an equal probability of similar exposure (i.e. similar dose and strain). Our results therefore strongly suggest the existence of individuals with a higher risk of developing CJD when exposed to TSE agents.

Our results suggest the existence of resistance to CJD among exposed heterozygotes, the basis of which remains to be explained. As we observed that genetic susceptibility in our iatrogenic CJD cases was similar to that reported in sporadic CJD, an environmental origin can be postulated. However, even if these TSE agents are highly resistant to degradation, the world-wide distribution of CJD remains difficult to explain: a logical explanation would be the existence of healthy human carriers. In the whole population, low levels of TSE agents would be pathogenic only in susceptible individuals. In this model, PrP<sup>3D</sup> would constitute a "virulence factor" for the agent. The existence of toxins which are encoded by micro-organisms and are responsible for their virulence is known in microbiology, as well as the usual presence of non-virulent forms of these micro-organisms in healthy humans. The original feature of TSEs is that the virulence factor appears to be a normal host protein deregulated by the agent. The normal proteolysis of PrP would be prevented, thus allowing PrP<sup>3D</sup> to accumulate in the cell. This neurotoxic PrP<sup>3D</sup> would allow the agent to escape regulation by the cell and to replicate efficiently. Resistance to infection would then depend on inability to accumulate the neurotoxic factor, which reflects a low level of PrP<sup>3D</sup> synthesis and/or inefficient modification of the normal PrP<sup>c</sup> catabolism. In this model, our results could be interpreted as follows. Heterozygosity at codon 129, leading to the synthesis of two forms of PrP with potentially different tertiary structures, may reduce the risk of self-aggregation or nucleation, which is one of the major mechanisms of resistance to degradation. In heterozygotes, the contaminating agent would not be able to convert PrP<sup>c</sup> efficiently into a protease-resistant isoform and would therefore not be pathogenic for the period of time studied. Conversely, in homozygotes the same contaminating agent would be able to induce the neurotoxic accumulation of PrP<sup>3D</sup>, thus allowing efficient replication of a virulent agent and the appearance of
CJD. This model ‘modified host protein as virulence factor’ is not incompatible with previous hypotheses and fits well with host susceptibility, the apparent toxicity of PrP<sup>CJD</sup> and the diversity of the strains of TSE agents reported.

At present, the iatrogenic cases so far reported in France seem to constitute about 6% of the children homozygous for PrP codon 129 who were given potentially contaminated batches of hGH (Job et al., 1992; Clément et al., 1992). This small percentage may merely indicate a rare, random and low level of contamination of the distributed hGH vials; on the other hand, it may represent only the first cases after massive contamination. We cannot exclude the possibility that this apparent low efficiency of disease development is not linked to further individual variation in genetic susceptibility to CJD. In that case, PrP codon 129 homozygosity would constitute only one parameter of this susceptibility.

We are now looking for other markers which can help to better define individuals highly susceptible to iatrogenic CJD. If the existence of groups at risk were confirmed, an experimental animal model would have to be established, to investigate the different factors responsible for pathogenesis and therefore for greater susceptibility. Furthermore, in such a model, it would be possible to investigate whether or not these genetic modifications make the animals more susceptible to agents originating from other species.

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**References**


