Prion protein in the cerebrospinal fluid of healthy and naturally scrapie-affected sheep

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The aim of this study was to characterize the cerebrospinal fluid (CSF) prion protein (PrP) of healthy and naturally scrapie-affected sheep. The soluble form of CSF PrP^C immunoblotted with an anti-octarepeat and an anti-C terminus mAb showed two isoforms of approximately 33 and 26 kDa, corresponding to the biglycosylated and unglycosylated isoforms of brain PrP^C. Neither the mean concentration nor the electrophoretic profile of CSF PrP differed between healthy and scrapie-affected sheep, whereas a slightly increased resistance of CSF PrP to mild proteolysis by proteinase K was evident in the CSF of scrapie-affected sheep. No difference in susceptibility to proteolysis was observed between the two ARR and VRQ genetic variants of the purified prokaryote recombinant PrP. It was concluded that the physicochemical properties of PrP^C in the CSF could be altered during scrapie and that these changes might reflect the physiopathological process of prion disease.

Prion diseases are characterized by neuropathological changes confined to the central nervous system and cerebral deposition of a partially proteinase K-resistant isoform (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^C), which is highly expressed in the brain. As cerebrospinal fluid (CSF) is the extracellular fluid that bathes the brain cells, the characteristics of PrP^C in this medium might reflect the physiopathological process affecting PrP^C in the brain. Indeed, PrP levels in the CSF might be altered by trapping of PrP in the PrP^{Sc} aggregates accumulated in the extracellular spaces. Alternatively, PrP^{Sc} molecules released by extracellular aggregates might join the interstitial fluid that is postulated to leak into the CSF. Different techniques have been developed to detect PrP^{Sc} in the CSF, but none has given consistent results (Bieschke *et al.*, 2000; Wong *et al.*, 2001). Although firm evidence of prion infectivity in the CSF is still lacking, studies based on experimental transmission suggest that the infectivity of CSF is low (Brown *et al.*, 1994).

We hypothesized that prion diseases might be associated with modifications of the CSF PrP concentration and/or biochemical properties that could be of physiopathological and diagnostic relevance. Ovine scrapie is a model of natural prion disease for which the impact of the prion protein gene (*PRNP*) polymorphism at positions 136, 154 and 171 on disease susceptibility is well documented. The $V_{136}R_{154}Q_{171}$ (VRQ) and ARR alleles have consistently been associated with the highest susceptibility or natural resistance to the clinical disease, respectively (Hunter *et al.*, 1997; Elsen *et al.*, 1999; Baylis *et al.*, 2004), although atypical scrapie strain(s) can naturally infect sheep harbouring the so-called resistant PrP genotype (Le Dur *et al.*, 2005).

Changes in PrP^C concentrations and/or biochemical features in the CSF of scrapie-affected sheep might reflect those occurring during spontaneous prion disease. Our aim was to evaluate the influence of scrapie on CSF PrP concentrations, electrophoretic profile and sensitivity to mild proteolysis by proteinase K (PK). Possible genetic involvement was examined by comparing the PK sensitivities of ARR and VRQ genetic variants of the purified recombinant ovine PrP expressed in *Escherichia coli*.

Table 1 gives the characteristics of the 13 clinically scrapie-affected and 12 healthy sheep used in the different experiments. PrP genotypes at codons 136, 154 and 171 were determined from blood samples (Scanelis, Toulouse, France). Scrapie diagnosis was confirmed by histological examination (neuronal and neuropil vacuolation in the grey matter, and gliosis) of the brainstem (obex). Immunohistochemical tests were performed in healthy sheep with no detectable histopathological lesions using a method described previously (Andréoletti *et al.*, 2000) to check for the absence of PrP^{Sc} deposition in the cerebellum, obex and cerebral cortex (O. Andréoletti, personal communication) and exclude possible atypical forms of scrapie.

All experimental procedures were performed in accordance with French legal requirements regarding the protection of laboratory animals. Lumbar CSF obtained by direct

Table 1. Characteristics of the sheep used in the different experiments

Code	Breed*	Gender	Genotype	Status	Experiment†
4680	M	Female	ARH/ARH	Healthy	ELISA
8887	M	Female	ARQ/ARH	Healthy	WB_{Pc248}
9397	M	Female	ARR/ARQ	Healthy	ELISA; WB_{Pc248}
R027	M	Female	ARR/ARQ	Healthy	ELISA; WB_{Pc248}
416	M	Female	ARQ/ARQ	Healthy	ELISA; WB_{Pc248}
083	M	Female	ARQ/AHQ	Healthy	ELISA; WB_{Pc248}
5969	M	Female	ARQ/ARH	Healthy	WB_{Pc248} (PK)
30096	R	Male	ARR/ARR	Healthy	ELISA; WB_{Pc248} (PK); WB_{BAR224} (PK); deglyc.
30104	R	Male	ARR/ARR	Healthy	ELISA; WB_{Pc248} (PK); WB_{BAR224} (PK)
30307	R	Male	ARR/ARR	Healthy	ELISA; WB_{Pc248} (PK); WB_{BAR224} (PK)
30308	R	Male	ARR/ARR	Healthy	ELISA; WB_{Pc248} (PK); deglyc.
30152	R	Male	ARR/ARR	Healthy	WB_{BAR224}
5356	M	Female	ARQ/ARQ	Scrapie	ELISA; WB_{Pc248}
9144	M	Female	ARH/VRQ	Scrapie	ELISA; WB_{Pc248}
1018	M	Female	ARQ/ARQ	Scrapie	ELISA
1017	M	Female	ARQ/ARQ	Scrapie	ELISA
0857	M	Female	ARQ/VRQ	Scrapie	WB_{Pc248}
487	M	Female	ARQ/ARQ	Scrapie	WB_{Pc248} (PK)
0830	M	Female	ARQ/ARH	Scrapie	WB_{Pc248}
0767	M	Female	ARQ/ARQ	Scrapie	WB_{Pc248}
0957	M	Female	ARQ/ARH	Scrapie	WB_{Pc248}
30028	R	Male	VRQ/VRQ	Scrapie	ELISA; WB_{Pc248} (PK); WB_{BAR224}; deglyc.
30200	R	Male	VRQ/VRQ	Scrapie	ELISA; WB_{Pc248} (PK); WB_{BAR224} (PK); deglyc.
30376	R	Male	VRQ/VRQ	Scrapie	ELISA; WB_{Pc248} (PK); WB_{BAR224} (PK)
30390	R	Male	VRQ/VRQ	Scrapie	WB_{Pc248} (PK)

*M, Manech Redface; R, Romanov.
†WB, Western blotting: the anti-PrP mAb used (Pc248 or BAR224) is indicated; PK is shown in parentheses when a PK test was performed; deglyc., deglycosylation.

puncture was centrifuged for 10 min at 1500 ***g*** and stored at −20 °C until assayed.

The electrophoretic profile of CSF PrP and sensitivity to mild proteolysis by PK were characterized by immunoblotting with two anti-PrP mAbs: Pc248, directed against the octarepeat region (Moudjou *et al.*, 2001), and BAR224, an anti-C terminus mAb recognizing residues 141–151 (Morel *et al.*, 2004). CSF samples were concentrated fivefold using a centrifugal device with a molecular mass cut off of 10 kDa (Nanosep; Pall Life Sciences). Approximately 15 μg protein from concentrated CSF and 6 μg protein from a 20 % brain homogenate in sucrose buffer from a healthy sheep were separated by SDS-PAGE using a Bio-Rad apparatus. Protein transfer was carried out onto a nitrocellulose membrane using a Minigel Transblot Cell System (Bio-Rad). The membrane was incubated with Pc248 or BAR224 anti-PrP mAbs at a 1 : 5000 dilution and the blots were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech) using goat anti-mouse IgG coupled to peroxidase (Amersham) at a 1 : 2000 dilution as the secondary antibody. Signal specificity was tested by incubating the membrane with the anti-mouse secondary antibody alone.

A deglycosylation experiment was also carried out to check that the protein bands that were immunodetected by mAb Pc248 in the ovine CSF corresponded to PrP glycoforms. Brain homogenate or unconcentrated CSF samples were treated with 2 U *N*-glycosydase F (Roche) and incubated overnight at 37 °C in the presence of 2 % Nonidet P40 and 1 % SDS. Samples were then diluted in Laemmli sample buffer and analysed by Western blotting.

The fivefold-concentrated CSF samples were treated with different concentrations of PK for 1 h at 37 °C. Proteolysis was stopped by adding SDS-sample buffer and heating for 5 min at 95 °C.

An ELISA approach was also developed to quantify and compare the PK sensitivities of PrP from the CSF of healthy and scrapie-affected sheep and those of genetic variants of the prokaryote recombinant ovine PrP (Rezaei *et al.*, 2000). Solutions (20 ng ml^{-1}) of the VRQ and ARR variants of the prokaryote recombinant ovine PrP were prepared in 0·1 M phosphate buffer containing 1 mg BSA ml^{-1}, 0·15 M NaCl and 0·01 % NaN_3. PK was added at final concentrations ranging from 0·125 to 3 μg ml^{-1} in CSF and from 0·004 to 0·125 μg ml^{-1} in the recombinant protein solutions.

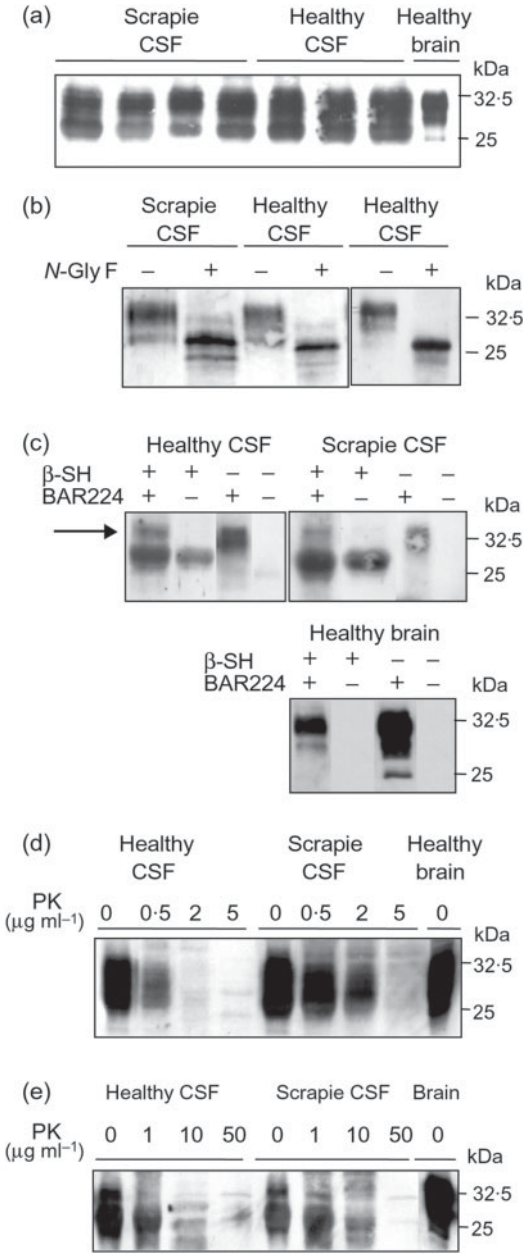

Fig. 1. Representative Western blots of PrP in CSF samples from scrapie-affected and healthy sheep. A brain homogenate from a healthy ewe was included for comparison. (a, b) Electrophoretic profile of CSF PrP detected by Western blotting with anti-PrP mAb Pc248 and effect of *N*-glycosydase F (*N*-Gly F) treatment on CSF PrP profile. (c) Electrophoretic profile of CSF PrP immunodetected with (+) or without (–) anti-PrP mAb BAR224 in reducing (β-mercaptoethanol; β-SH) (+) or in non-reducing (–) conditions. The arrow indicates the biglycosylated form of PrP. Note that the reactivity of mAb BAR224 is enhanced in non-reducing conditions. (d, e) PK sensitivity of CSF PrP. Samples were incubated with or without PK at final concentrations of 0·5, 2 or 5 μg ml^{-1} (d) or 1, 10 or 50 μg ml^{-1} (e). Immunoblots were visualized with anti-PrP mAb Pc248 (d) or with anti-PrP mAb BAR224 (e). The sizes of the molecular mass markers (New England Biolabs) are given on the right of each panel.

Reactions were carried out at 37 °C for 1 h, stopped with 4 mM stopper (Pefabloc; Roche) and analysed by ELISA using anti-PrP antibodies SAF34 and 12F10-AchE (SPI-BIO). The whole CSF protein concentration was determined by Bradford's method, using a Bio-Rad protein assay kit.

Results were reported as mean ± SEM. Statistical analyses were performed using SYSTAT 8.0 (SPSS Inc.). Proteolysed PrP^C concentrations expressed as a percentage of the PrP^C concentration in untreated CSF were determined from the difference between the PrP^C concentration in untreated CSF and uncleaved PrP^C concentrations evaluated by ELISA. The relationship between PK and the proteolysed PrP fraction was determined by plotting the proteolysed PrP fraction against the PK concentrations. The data were fitted by applying the sigmoidal Hill model, as described previously (Toutain, 2002). The parameters E_{max} (100 %), EC_{50} (median effective concentration; potency) and φ (slope) were evaluated using a non-linear least-squares regression program and the effect of scrapie on mean parameters was analysed by Student's *t*-test.

The effect of the genetic variant of the prokaryote recombinant PrP on uncleaved PrP concentrations was analysed using a two-factor ANOVA (variant and PK concentration effects).

The CSF PrP^C profile of healthy sheep on a Western blot probed with mAb Pc248 (Fig. 1a) showed two major bands migrating at approximately 33 and 26 kDa, at about the same level as the biglycosylated and unglycosylated isoforms of brain PrP^C, respectively. CSF treatment with *N*-glycosydase F generated two immunoreactive bands, one major band of 26 kDa, corresponding to the full-length non-glycosylated PrP^C isoform, and another less reactive band with a reduced molecular mass of 23 kDa (Fig. 1b). As this band was recognized by an anti-octarepeat mAb (Pc248), it might correspond to a PrP C-terminal truncated form, as also observed in fluids from the genital tracts of sheep (Ecroyd *et al.*, 2004). On the Western blot probed with mAb BAR224, the PrP profile in the CSF showed one thin, specific band migrating approximately the same distance as the 33 kDa biglycosylated PrP^C from the brain (Fig. 1c). The major band detected in CSF at 27 kDa corresponded to the endogenous IgG light chains cross-reacting with the secondary antibody, as it was systematically detected in the absence of primary antibody and disappeared in non-reducing conditions. This band was not detected in the control brain sample (Fig. 1c). A low affinity of BAR224, in comparison with Pc248 mAb, for PrP could explain the PrP profile obtained with mAb BAR224. The immunoreactive PrP profiles for the two anti-PrP antibodies did not differ between healthy and scrapie-affected sheep (Fig. 1a and c).

On the Western blot probed with mAb Pc248, PrP^C was completely digested by treatment of CSF with 5 μg P Kml^{-1} in both healthy and scrapie-affected sheep (Fig. 1d). Nevertheless, the fraction of PrP that remained uncleaved

with 0·5 and 2 μg PK ml^{-1} was greater in the CSF of scrapie-affected sheep than in that of healthy ones. When probed with the anti-C terminus mAb BAR224, a positive PrP signal was still observed followed digestion with up to 1 μg PK ml^{-1} in scrapie (Fig. 1e) but not in healthy CSF. This signal disappeared in both healthy and scrapie CSF after treatment with 10 μg PK ml^{-1} (Fig. 1e). The faint band detected after treatment with 50 μg PK ml^{-1} might correspond to PK, which is known to give a faint positive signal with ECL reagents.

Physiopathological status had no effect on whole-protein concentrations in the CSF (176·7±29·6 vs 232·7±62·5 μg ml^{-1} in nine healthy vs seven scrapie-affected sheep, $P>0·05$). The PrP isoform monitored by ELISA might correspond to uncleaved PrP, as SAF34, an anti-octarepeat region mAb, was used for capture in the ELISA experiments. The mean CSF PrP concentration of scrapie-affected sheep (10·9±1·3 ng ml^{-1}, $n=7$) did not differ from that of healthy sheep (9·6±0·81 ng ml^{-1}, $n=9$).

Fig. 2 illustrates the relationship between the fraction of proteolysed PrP and PK concentrations in the CSF of representative healthy and scrapie-affected sheep and the corresponding fitted curves. The mean EC_{50} value of PK determined from the CSF of scrapie-affected sheep was greater than that evaluated from the CSF of healthy sheep (0·51±0·05 vs 0·35±0·05 μg ml^{-1}, $P<0·05$), indicating that the relative resistance of CSF PrP to mild proteolysis by PK was increased in scrapie-affected sheep. The concentration of the two ARR and VRQ genetic variants of the prokaryote recombinant PrP decreased exponentially with PK concentration, reaching undetectable values at 0·125 μg ml^{-1}, and did not differ, whatever the PK concentration (data not shown).

CSF PrP concentrations from scrapie-affected sheep did not differ from those of healthy ones. As observations in Alzheimer's disease have shown that the level of soluble amyloid decreases whereas the amount of protease-resistant

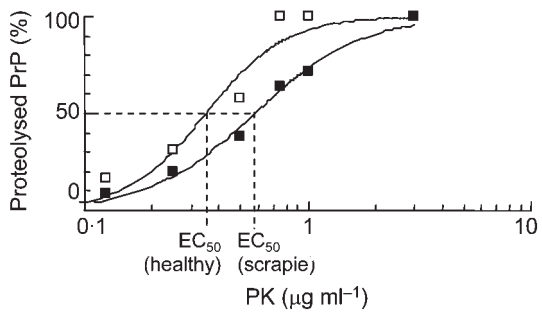

Fig. 2. Semi-logarithmic plot of observed and fitted fraction (solid line) of CSF PrP proteolysed as a function of PK concentrations in a representative healthy (□) and a representative scrapie-affected sheep (■). EC_{50} is the PK concentration required for 50 % proteolysis.

β-amyloid increases (Weller, 1998), one might have expected a fall in soluble PrP levels in the CSF of scrapie-affected sheep resulting from the formation of amyloid deposits. In our study, mature CSF PrP represented only 0·01 % of the total proteins, i.e. a ratio within the same range as that reported for PrP^C in the nervous tissue (Moudjou *et al.*, 2001). PrP^C in the CSF could originate from the brain, being released by cells lining the ventricular system (ependyma, choroid plexus) and/or by neuronal cells in the interstitial fluid, the contents of which are postulated to leak into the CSF (Weller, 1998). However, it cannot be excluded that a portion of the PrP^C might be transported from the blood to the CSF by pinocytosis or specific carrier mechanisms at the level of the choroid plexus. Indeed, a soluble form of PrP^C was detected in ovine plasma (data not shown) and 80 % of the proteins found in the CSF were shown to originate from the systemic circulation (Green, 2002). This is important for estimating the risk of transmission of prion disease via blood transfusion, as PrP^{Sc} transported from the blood to the CSF could diffuse across the ependyma lining the ventricular system into the brain tissue. Banks *et al.* (2004) have shown that the murine scrapie prion protein can cross the blood–CSF barrier and enter the brain CSF compartment.

The PrP isoforms monitored in the CSF were PK sensitive and no intermediate degradation products could be evidenced by immunoblotting. When probed with the anti-C terminus mAb BAR224, the specific signal was abolished in CSF from healthy and scrapie-affected sheep at PK concentrations of about 10 μg ml^{-1}, corresponding to 5 μg (mg protein)$^{-1}$, i.e. the same PK concentration range used for diagnostic purposes (Baron *et al.*, 1999). These results strengthen the hypothesis that PrP^{res} is absent in the CSF of scrapie-affected sheep.

Our results also showed that PrP in the CSF of scrapie-affected sheep was 'more resistant' than PrP from the CSF of healthy sheep to proteolysis by PK. Although slight, this effect was robust and reproducible. Indeed, it was demonstrated both by immunoblot with two anti-PrP mAbs that bind very distinct regions of the protein and by ELISA. This differential PK effect could originate from intrinsic variations in the PK sensitivity of the protein variants, as all scrapie-affected sheep except four were homozygous for VRQ or ARQ alleles encoding susceptible variants of PrP, whilst more than half of the healthy sheep carried one copy of the ARR or AHQ alleles conferring resistance. Buschmann *et al.* (1998) have shown that the PrP^C of brain homogenates of homozygous ARR/ARR healthy sheep display a higher sensitivity to PK than the PrP^C of healthy ARQ/ARQ sheep. Such a difference was also observed when the two ARR and VRQ variants of the prokaryote recombinant ovine PrP were compared (Rezaei *et al.*, 2000). In our experimental conditions, the recombinant PrP protein was highly sensitive to PK and no difference in sensitivity to PK was evidenced between the two genetic variants. This suggests that the higher resistance of PrP from

the CSF of scrapie-affected sheep may not result from *PRNP* gene polymorphism. Instead, the slower decrease in uncleaved PrP with increasing PK concentrations in the CSF of scrapie-affected sheep could reflect a partial change in the physicochemical properties of PrP, i.e. a transitional state of PrP towards a pathological isoform in scrapie-affected sheep, which could be of physiopathological significance. In agreement with this hypothesis, Safar *et al.* (1998) have suggested the existence of an intermediate protease-sensitive isoform of the prion protein formed during the process of transconformation of PrP^{C} into PrP^{Sc}.

In conclusion, we have shown that a soluble PrP is present as a protease-sensitive isoform in the CSF of healthy and scrapie-affected sheep. The PrP concentrations in CSF and the electrophoretic profile were unaffected by the disease. However, our results suggest that the biochemical feature of PrP, i.e. its relative resistance to proteolysis, is slightly modified in scrapie-affected sheep.

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