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Oxidative Damage to Nucleic Acids in Human Prion Disease

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Recently, several studies proposed a physiological role for the cellular prion protein (PrP^c) in defense against oxidative stress. Since the pathogenesis of prion disease necessarily involves a disturbance of PrP^c homeostasis, we hypothesized that such diseases would be associated with concomitant disturbances in oxidative balance. In support of such a notion, in this study we show increased oxidative damage to nucleic acids in affected brains of patients with Creutzfeldt–Jakob disease. These data suggest that damage by free radicals is a likely cause for neurodegeneration in human prion disease, and antioxidants are a potential therapy for these disorders. Further, our data support the hypothesis that loss of the anti-oxidant function of PrP^c plays a key role in the pathogenesis of these disorders.

INTRODUCTION

Transmissible spongiform encephalopathies or prion diseases are infectious, inherited or sporadic neurodegenerative disorders (for a review see (Prusiner, 1998)). Human prion diseases include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease, kuru and fatal familial insomnia (FFI). The infectious agent (prion) in prion disease, different from both viroids and viruses (Prusiner, 1982), is considered to be formed by an abnormal isoform (PrP^{Sc}) of the host-encoded prion protein (PrP^c). However, the biological basis of disease manifestation and cell loss remains unknown and likely involves either loss of function for PrP^c (Collinge *et al.,* 1994) or neurotoxicity of accumulated PrP^{Sc} (Forloni *et al.,* 1993). PrP^c is a copper binding (Brown *et al.,* 1997a), membrane-attached GPI-anchored glycoprotein characterized by a high degree of amino acid sequence conservation within mammals (Prusiner, 1998).

There is increasing evidence obtained by morphological and biochemical examination of diseased brains, that oxidative stress, resulting from damage by free radicals to lipids, carbohydrates, proteins, and nucleic acids, is involved in neurodegenerative disorders as Alzheimer's disease (Nunomura *et al.,* 1999a, 2000; Smith *et al.,* 1997), Down's syndrome (Nunomura *et al.,* 2000), amyotrophic lateral sclerosis (Cookson and Shaw, 1999), Parkinson's disease (Zhang *et al.,* 2000), and Huntington's disease (Browne *et al.,* 1999). These investigations have led to a better understanding of the pathogenesis of neurodegeneration and raised hopes for the development of novel (antioxidant) therapeutic approaches (Rottkamp *et al.,* 2000).

Recently, others and we have indicated a key role for PrP^c in the cellular response and resistance to oxidative stress. Neurons and astrocytes from mice deficient in PrP^c were shown to be more sensitive to oxidative stress (Brown *et al.,* 1997c, 1998; White *et al.,* 1999) and PrP^c has an activity like that of superoxide dismutase (Brown *et al.,* 1999; Wong *et al.,* 2000). Further, in mice devoid of prion protein, higher levels of oxidative damage to proteins and lipids and induction

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of heme oxygenase-1 and neuronal nitric oxide synthase was detected (Wong *et al.*, 2001a,b). Moreover, oxidative stress has been shown to increase PrP^c expression in cultured human lens epithelial cells (Frederikse *et al.*, 2000) and to favor the formation of the unglycosylated form of the prion protein which increases the risk of conversion of PrP^c to PrP^{Sc} (Capellari *et al.*, 1999). A marked increase of neuronal prion protein expression was recently shown in neurodegeneration, including prion diseases and AD (Voigtlander *et al.*, 2001). Taken together these data suggest a direct role of PrP^c in the cellular resistance to oxidative stress. In this study we test on human tissue material the hypothesis that oxidative stress occurs in prion diseases.

MATERIALS AND METHODS

Tissue. Brain tissue was obtained at autopsy from clinically and neuropathologically confirmed cases of prion diseases: age range 37–73 years, median 67; postmortem interval 5–120 h, median 30.5, $n = 14$ (2 FFI, 2 familial CJD and 10 sporadic CJD) (Table 1) and was compared with tissue from nondemented control cases (age range 53–78 years, median 65; postmortem 14–27 h, median 20, $n = 9$) and definitive AD cases (age range 61–85 years, median 80; postmortem 6–24 h, median 20.5; $n = 5$) according to the CERAD criteria (Khachaturian, 1985), with similar postmortem intervals before fixation. Cause of death in the control cases was coronary failure (3 cases), idiopathic pulmonary fibrosis and cardiomyopathy (1), aneurysm bleeding (1), B-cell lymphoma (2), acid ingestion (1), and hepatoma (1). Blocks from the hippocampus and adjacent temporal cortex including CA4, CA3, CA2, CA1, subiculum (SUB), praeparasubiculum (PrS), entorhinal cortex (EC), and temporal cortex (TC) were studied. These regions were selected because they include, within one tissue block, areas that have maximum tissue damage as well as areas with little or no lesioning in CJD (Guentchev *et al.*, 1997).

Immunocytochemistry and antibodies. All sections were cut at 4 μm. After deparaffinization with xylene, the sections were hydrated through graded ethanol. Endogenous peroxidase activity in the tissue was eliminated by incubation with 3% H_2O_2 in methanol, and nonspecific binding sites were blocked.

To detect oxidized nucleosides immunocytochemistry was performed as reported previously (Nunomura *et al.*, 1999a). In brief: we used a monoclonal antibody developed by immunizing mice with 8-hydroxyguanosine (8-OHG): 1F7 (1:30, Trevigen, Gaithersburg, MD). The antibody recognizes RNA-derived 8-OHG as well as DNA-derived 8-hydroxydeoxyguanosine (8-OHdG); its sensitivity and specificity, as well as validity as indicator of nucleic acid oxidative damage, were published (Nunomura *et al.*, 1999a, 2000). For 1F7 the sections were pretreated with proteinase-K (10 μg / ml in PBS, pH 7.4, for 40 min at 37°C; Boehringer-Mannheim, Indianapolis, IN). The specificity of the immunoreactivity was tested as reported previously (Nunomura *et al.*, 1999a, 2000). After the proteinase-K treatment, additional sections were pre-

TABLE 1
Clinical Data and Summary of the Semiquantitative Analysis of 8-OHG / 8-OHdG Staining

	Sex	Duration	Age	8-OHG / 8-OHdG score	*PRNP*	
CJD						
1	F	12	76	+++ (*)		M129M
2	M	9	62	+++ (*)		M129M
3	F	3	75	+++ (*)	E200K	M129M
4	F	12	67	+++ (*)		na
5	M	3	61	++ (*)		na
6	M	6	61	++	D178N	V129V
7	F	6	67	++		M129M
8	F	1	70	+		na
9	F	2	46	−/+		na
10	F	2	72	−/+		M129V
11	M	4	85	−		M129M
12	F	4	73	−		M129V
FFI						
1	M	4	37	−	D178N	M129M
2	M	13	70	−	D178N	M129M
AD						
1	F		80	+++		
2	M		61	++		
3	M		85	++		
4	M		80	+		
5	M		82	+		
Control						
1	F		53	+		
2	M		65	−/+		
3	F		55	−/+		
4	F		56	−/+		
5	M		75	−/+		
6	M		76	−/+		
7	M		55	−/+		
8	F		74	−		
9	F		78	−		

Note. Lack of neuronal staining (−), weak (+/−), moderate (+), strong (++), and very strong (+++); *PRNP*, prion protein gene; (*) presence of conglomerates.

treated with DNase I (10 U/μl in PBS for 1 h at 37°C; Boehringer-Mannheim), S1 DNAse (10 U/μl in PBS for 1 h at 37°C; Boehringer-Mannheim), RNAse (5 μg/μl in PBS for 1 h at 37°C; Boehringer-Mannheim) before incubation with 1F7.

Two different anti-PrP antibodies were used for immunocytochemical detection of PrP^{sc}: (1) mouse anti-hamster PrP (clone 3F4, 1:500, Senetek PLC, Maryland Heights, Missouri) and (2) mouse anti-human PrP (clone 6H4, 1:500, Prionics, Zurich). The sections were hydrated-autoclaved at 121°C for 30 min, followed by incubation in 98% formic acid for 1 min and incubation in guanidine thiocyanate at 4°C for 2 h.

Mouse anti-Tau (clone AT-8, 1:200, Innogenetics, Belgium) and mouse anti-Aβ (clone 6F/3D 1:50, Dako, Glostrup, Denmark) antibodies and a rabbit anti-GFAP (1:400, Dako, Glostrup, Denmark) antiserum were used to detect astrogliosis and Alzheimer's disease pathology. Sections for GFAP staining were pretreated with 98% formic acid for 1 min.

Immunostaining was developed by the peroxidase-antiperoxidase procedure, using 0.75 mg/ml 3,3′-diaminobenzidine cosubstrate in 0.015% H_2O_2 and 50 mM Tris–HCl, pH 7.6, for 10 min or with ChemMate detection kit (Dako, Glostrup, Denmark) according to the manufacturer's protocol.

Semiquantitative evaluation of 8-OHG/8-OHdG staining was made as follows: lack of neuronal staining was graded as (−), weak as (+/−), moderate as (+), strong as (++), and very strong as (+++). Since (if present) positive cells appeared in large numbers the evaluation was made on basis of the intensity of staining. The semiquantitative analysis was performed independently by two of us in a blinded fashion (M.G. and S.S.). Both obtained similar results.

RESULTS

Most (8/12) CJD cases showed very strong (+++), strong (++), or moderate (+) immunostaining for oxidized nucleotides (Table 1) in the cytoplasm of a majority of the neurons (Figs. 1C, 1D, and 2). Immunoreactivity was predominantly localized in the neuronal cytoplasm, whereas neuronal nuclei showed weaker staining. In three cases some nuclei of cytoplasmically unstained neurons were 8-OHG/8-OHdG immunoreactive (data not shown). The neuropil was spared. The 8-OHG/8-OhdG positive neurons were mainly localized in the temporal cortex (Fig. 1), while hippocampal neurons appear to be spared. After DNase pretreatment the nuclear staining was significantly diminished and the cytoplasmic staining was abolished. After RNAse pretreatment the cytoplasmatic staining was weaker (data not shown).

There was no unequivocal difference between familial and sporadic CJD cases (Table 1). Cases with longer duration of disease had a stronger staining for oxidized nucleotides: cases with very strong (+++) to moderate (+) staining had an average duration of 7.3 months, and cases with weak (−/+) or no (−) staining 2.5 months. In five CJD cases (Table 1) we found groups of very strongly stained cortical neurons (Figs 2A and 2B). These groups were composed by variable numbers (2 to >50) of 8-OHG/8-OHdG-positive cells with neuronal morphology. No astro- or microglial appearing cells in between the strongly positive neurons were stained (Figs. 2A and 2B). These neuronal conglomerates were localized only in the cerebral cortex. They were not restricted to a particular layer.

All CJD cases were positive for PrP^{Sc}. There was no regional correlation between distribution of PrP^{Sc} or spongiform change and 8-OHG/8-OHdG immunoreactivity (Fig. 1). The two FFI cases showed no or only weak staining for oxidized nucleotides (Fig. 1). There was also no detectable PrP^{Sc} in investigated regions (Fig. 1).

In most control brains (8 from 9) there was no (−) or only weak (−/+) neuronal (cytoplasmatic) and background staining (Fig. 1I). In one from nine control cases the neuronal staining was moderate (+); this patient had died from chronic idiopathic pulmonary fibrosis and cardiomyopathy. None of the controls showed very strong or strong staining for oxidized nucleotides. The weakly stained neurons were mainly pyramidal neurons in the CA4-1 area of the hippocampus. PrS, EC, and TC were almost free of 8-OHG/8-OHdG immunoreactive neurons. In a few cases, weak staining of astrocytic nuclei was observed. In none of the control cases was PrP^{Sc} detected.

In AD brains, we found variably intensity of immunolabeling for oxidized nucleosides (8-OHdG and 8-OHG) in the neuronal cytoplasm (Table 1 and Fig. 1L). Cytoplasmic immunoreaction was on granular structures with a Nissi substance-like distribution extending from the cell bodies to dendrites, but with no long processes displaying the immunoreaction (Fig. 1L). Intranuclear immunoreaction was very weak or absent. Immunoreactivity in AD brains resembled the pattern in controls; however, it was more intense (Table 1). The presence of oxidized nucleosides in neurons was not related to the presence of neurofibrillary tangles or apposition to senile plaques. There was no detectable PrP^{Sc} in AD brains.

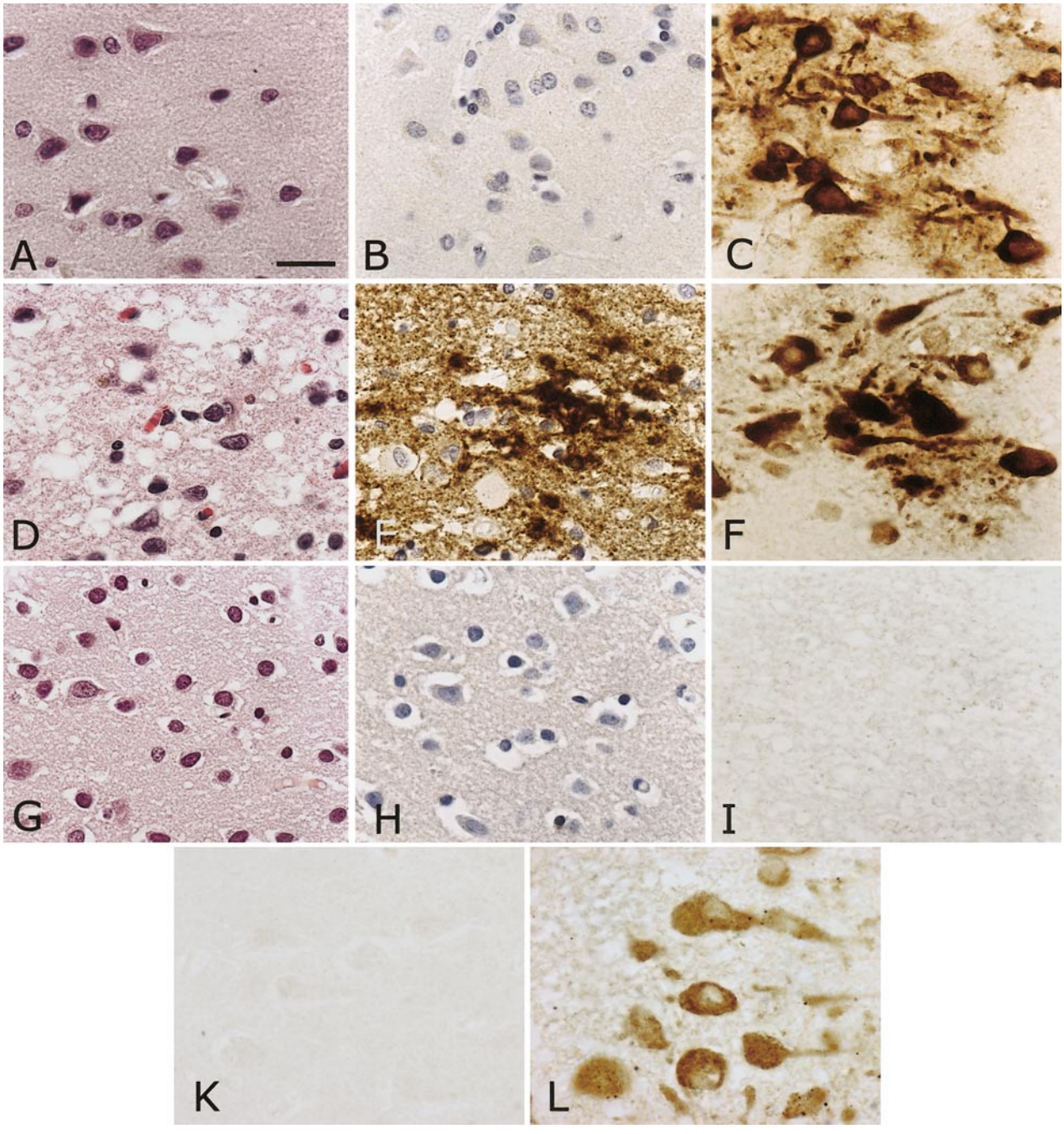

FIG. 1. HE-stained sections (A, D, G) and immunostaining for PrP^{Sc} (B, E, H) and 8-OHG/8-OHdG (C, F, I, K, L) in a CJD (A, B, C, D, E, F), FFI (G, H, I), control (K), and AD (L) case. Note that one of the CJD cases has almost no detectable PrP^{Sc} deposition (B) and spongiform change in the temporal cortex (A), and the other vice versa (D, E). Remarkably both cases have OHG/8-OhdG immunoreactive neurons. Bar, 25 μm.

DISCUSSION

This study for the first time shows increased labeling for an oxidative stress marker in brains of CJD patients, thus suggesting that oxidative damage is associated with the pathology of prion disease. The data we describe here are in accordance with previous reports showing increased levels of oxidative stress markers detected in the brains of scrapie mice (Guentchev *et al.*, 2000) and mice devoid of prion

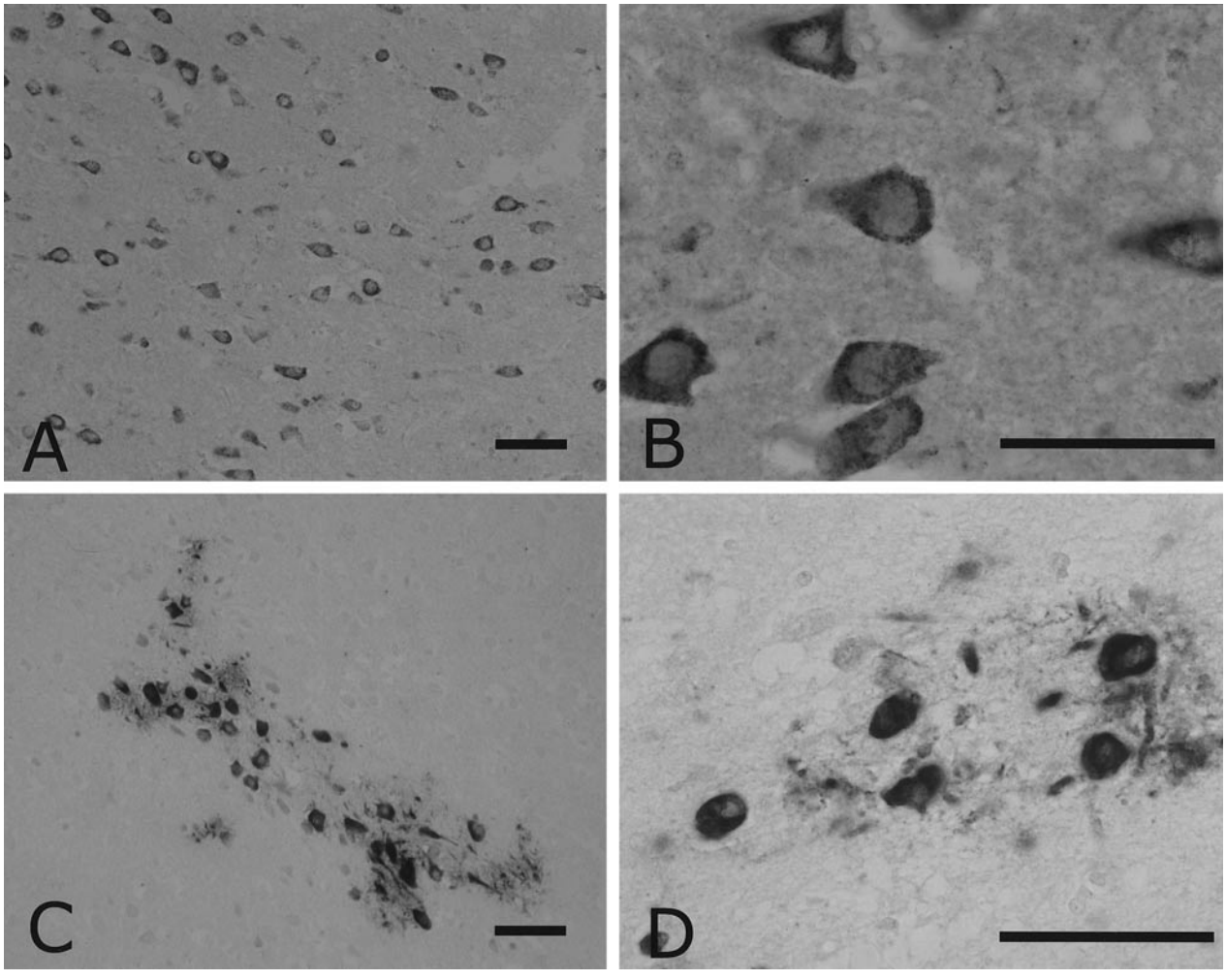

FIG. 2. Immunostaining for 8-OHG/8-OHdG in 2 CJD cases. One showing a diffuse staining (A and B), the other (C and D) conglomerates. Bar, 25 μm.

protein (Wong *et al.*, 2001a). The fact that both enzymes RNAse and DNase were able to abolish the immunostaining suggests that in human prion disease free radicals damage both DNA and RNA.

In some (5/12) CJD brains we found neuronal-group (conglomerate) staining pattern which is not seen in controls or AD (Nunomura *et al.*, 1999a, 1999b, 2000). Interestingly, in these conglomerates only cells with neuronal morphology and not others (astro-, microglial appearing cells) are stained. However, the interpretation of this finding is difficult. The presence of 8-OHG/8-OHdG positive neuronal conglomerates, some composed by 2–3 other by more than 50 neurons, might mean that the neurodegeneration starts multifocally in groups of cells which tend to recruit additional neurons.

It is not surprising that we failed to detect any oxidized nucleotides in FFI, since FFI differs clearly from other human prion disease by showing only slight neuronal loss and no detectable PrP^{Sc} in the hippocampus and adjacent cortex (Guentchev *et al.*, 1999). Further studies on FFI cases should investigate whether there are products of oxidative damage in brain regions with severe pathology (e.g., thalamus). Worth mentioning is the fact that only the control case which died from pulmonary fibrosis and cardiomyopathy had increased 8-OHG/8-OHdG immunoreactivity. We suggest that the changes observed are not a variation of the normal, but more likely are a consequence of the premortal progressive hypoxic condition of the patient (Halliwell, 1992; Hayashi *et al.*, 1999).

Although a great deal of progress has been made investigating the pathogenesis in prion diseases in past decades (Bueler *et al.*, 1993; Prusiner, 1998; Sailer *et al.*, 1994; Telling *et al.*, 1995), the mechanism of neurodegeneration is still an enigma. Several studies have shown that fragments of the prion protein are neurotoxic (Forloni *et al.*, 1993). This finding indicated that cerebral accumulation of PrP^{Sc} and its degradation products may be the cause for neurodegeneration in prion diseases. On the other hand, other investigators have shown an important function of PrP^{c} in synaptic transmission (Collinge *et al.*, 1994), in oxidative stress defense (Brown *et al.*, 1997b,c, 1999) and in long time survival of cerebellar neurons (Sakaguchi *et al.*, 1996). Taken together, these data suggest loss of the cellular function of PrP^{c} is an important event in prion diseases.

In this study we propose that oxidative damage to nucleic acids is a possible mechanism of degeneration in human prion diseases. The most likely source of oxidized nucleosides is from hydroxyl radicals formed from the reaction of highly diffusible H_2O_2 with redox-active metals as copper (Schubert and Wilmer, 1991). Since PrP^{c} binds copper (Brown *et al.*, 1997a) and its concentration correlates with resistance to oxidative stress (Brown *et al.*, 1997b), our data are supportive of a possible role of oxidative stress in cell death in prion diseases. Studies on brains of patients with Down syndrome suggest that increased levels of oxidative damage occur prior to the onset of Aβ deposition (Nunomura *et al.*, 2000), suggesting that oxidative stress is a pathognomonic event. The detailed time course of the pathologic changes described in this study have to be studied in an experimental model.

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