Acta Neuropathol (2005) 110: 232–238
DOI 10.1007/s00401-005-1034-4

REGULAR PAPER

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Redox metals and oxidative abnormalities in human prion diseases

Received: 1 March 2005 / Revised: 18 April 2005 / Accepted: 18 April 2005 / Published online: 11 August 2005

Abstract Prion diseases are characterized by the accumulation of diffuse and aggregated plaques of protease-resistant prion protein (PrP) in the brains of affected individuals and animals. Whereas prion diseases in animals appear to be almost exclusively transmitted by infection, human prion diseases most often occur sporadically and, to a lesser extent, by inheritance or infection. In the sporadic cases (sporadic Creutzfeld-Jakob disease, sCJD), PrP-containing plaques are infrequent, whereas in transmitted (variant CJD) and inherited (Gerstmann-Straussler-Scheinker Syndrome) cases, plaques are a usual feature. In the current study, representative cases from each of the classes of human prion disease were analyzed for the presence of markers of oxidative damage that have been found in other neurodegenerative diseases. Interestingly, we found that the pattern of deposition of PrP, amyloid-β, and redox active metals was distinct for the various prion diseases. Whereas 8-hydroxyguanosine has been shown to be increased in sCJD, and inducible NOS is increased in scrapie-infected mice, well-studied markers of oxidative damage that accumulate in the lesions of other neurodegenerative diseases (such as Alzheimer's disease, progressive supranuclear palsy, and Parkinson's disease), such as heme oxygenase-1 and lipid peroxidation, were not found around PrP deposits or in vulnerable neurons. These findings suggest an important distinction in prion-related oxidative stress, indicating that different neurodegenerative pathways are involved in different prion diseases.

Keywords Creutzfeld-Jakob disease · Gerstmann-Straussler-Scheinker syndrome · Oxidative damage · Prion · Redox metals

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Introduction

Prion diseases are associated with a conformational change in the structure of a normal cellular protein, the prion protein (PrP^{C}), which results in the production of a pathogenic protein form that is referred to as PrP^{SC} [28]. The properties associated with this structural

change include insolubility in non-ionic detergents, resistance to digestion with proteases, and the formation of fibrils after extraction in non-ionic detergents. The protease resistance of PrP^{SC} has been used extensively in the diagnosis of prion diseases [21].

Prion diseases affect a variety of organisms ranging from experimentally infected mice to humans [11, 28]. However, only in humans has the full range of disease acquisition been demonstrated: sporadic conversion, inheritance, or infection [9]. When propagated by infection, prion diseases are usually associated with PrP-amyloid plaques. Thus, animal diseases such as scrapie, bovine spongiform encephalopathy (BSE), and chronic wasting disease (CWD), which are transmitted by infection, usually exhibit deposition of PrP in plaques. Interestingly, some scrapie strains develop plaques in mice, i.e., 87V, and variant Creutzfeld-Jakob disease (vCJD), which has been associated with the ingestion of contaminated beef, usually exhibits high levels of PrP protein-containing-plaques [18] as do the human diseases Kuru [20] and Gerstmann-Straussler-Scheinker syndrome (GSS). On the other hand, in the most common form of prion disease in humans, sporadic CJD (sCJD), only ~5–10% of cases show plaques [6], and fatal familial insomnia and sporadic fatal insomnia are both devoid of plaques [2, 6].

Recently, there has been a great deal of interest in the role of transition metals in oxidative stress and neurodegenerative disease [24, 26, 36]. Since there is a well-described association between the PrP and copper, we and others have speculated that metal interactions and oxidative stress may, like in other neurodegenerative disorders, be critical to prion diseases [1, 5, 33, 38, 39]. In the current study, we examined metal deposition in several categories of human prion diseases, sCJD, GSS and vCJD (Table 1). In sCJD, redox metal deposition was never associated with PrP deposition. Conversely, in GSS and vCJD, metal deposition is invariably associated with PrP deposits, even in the absence of amyloid-β deposition. The oxidized nucleic acid base, 8-hydroxyguanosine (8OHG), previously found increased in sCJD [14], is also increased in cortical neurons in GSS along with mitochondrial (mt) DNA deletions. Other reactive oxygen species, lipid peroxidation, heme oxygenase-1 (HO-1) induction, and glycation were not detected in sCJD and were only weakly associated with PrP deposits in GSS. These findings suggest a distinction in oxidative stress and metal accumulation in prion conditions marked by whether PrP deposition is a constant feature.

Materials and methods

Tissue samples

The cases used in this study are outlined in Table 1. Tissue used included cases of GSS, Indiana 198 kindred ($n = 4$, ages 49–77 years) [3, 27], vCJD ($n = 3$, ages 25, 28, 29 years) [18], sCJD ($n = 19$, ages 36–80 years, including a series of cases categorized by subtype) [9]. Tissue was fixed in either formalin or the non-aldehyde fixatives, Carnoy's or methacarn, dehydrated, and embedded in

Table 1 Case information (*CJD* Creutzfeld-Jakob disease, *GSS* Gerstmann-Straussler-Scheinker syndrome)

	Age (years)	Duration (months)	Genotype	Fixative
Sporadic CJD	66	3		Carnoy
	54	12	MV	Carnoy
	80	5	MV mixed	Carnoy
	63	3		Carnoy
	66	2		Methacarn
	55	2		Methacarn
	56	4		Methacarn
	52	4		Methacarn
	61	5	MV mixed	Carnoy
	79	4	MM1	Carnoy
	66	1.5	MV1	Formalin
	70	2	MM1	Formalin
	71	2	MM1	Formalin
	62	3		Formalin
	58	3.5	VV2	Formalin
	67	3.5	VV1	Formalin
	74	6	MV1	Formalin
	36	18	VV2	Formalin
	62	9	MV2	Formalin
Variant CJD	25	10	M/M	Formalin
	28	9	M/M	Formalin
	29	10	M/M	Formalin
GSS	49	7.2	Indiana 198	Carnoy
	60	161	Indiana 198	Formalin
	77	106	Indiana 198	Formalin
	63	109	Indiana 198	Formalin

paraffin. Samples of cortex, hippocampus and cerebellum were studied from cases of sCJD, cerebellum and hippocampus were analyzed from the GSS cases, and cortex and cerebellum were analyzed from vCJD.

Immunocytochemistry

Sections were cut at 6 μm and placed on coated slides. Immunocytochemistry was performed using the peroxidase anti-peroxidase technique with 3,3'-diaminobenzidine as substrate. Microwave treatment in HCl was used for localization of prion deposits with monoclonal antibody 3F4 [19]. Other markers used included polyclonal antisera against HO-1 [35], hydroxynonenal (HNE) pyrrole adducts [31]), and carboxymethyllysine (CML) [4], and monoclonal markers against amyloid-β (4G8, Senetek) [25], cytochrome oxidase 1 (COX-1, Molecular Probes) [16], 8OHG (Trevigen) [14], and antiserum to ferritin (Dako).

Redox metals

Sites of redox-active iron were detected by incubation in 7% potassium ferrocyanide in 3% HCl and followed by detection with diaminobenzidine and H_2O_2 as cosubtrates (modified Perl stain) [36].

mtDNA deletions

Some sections were used to detect the 5-kb deletion in mtDNA by in situ hybridization [16].

Results

Gerstmann-Straussler-Scheinker syndrome

Two sites of oxidative damage, namely PrP plaque and vacuolar, were noted in the cases studied, and these varied according to disease condition. In GSS, a great number of prion-positive deposits were found in the cerebellum and hippocampal sections, many with a central core. Striking is the finding of redox-active iron in the majority of the cored prion deposits and, in some cases, within the presumed glia cells surrounding the accumulations. Ferritin is also found associated with some of the PrP deposits, and more strikingly, glial cells prominent in affected areas (Fig. 1). Hippocampal and temporal cortical neurons showed high levels of neuronal 8OHG and mtDNA accumulation (Fig. 2). Heme oxygenase-1 and CML are present around PrP deposits of GSS, although at low levels (not shown). Prion deposits, however, did not display amyloid-β (4G8), but the neurons did display increased phosphorylated tau [3, 10] (data not shown).

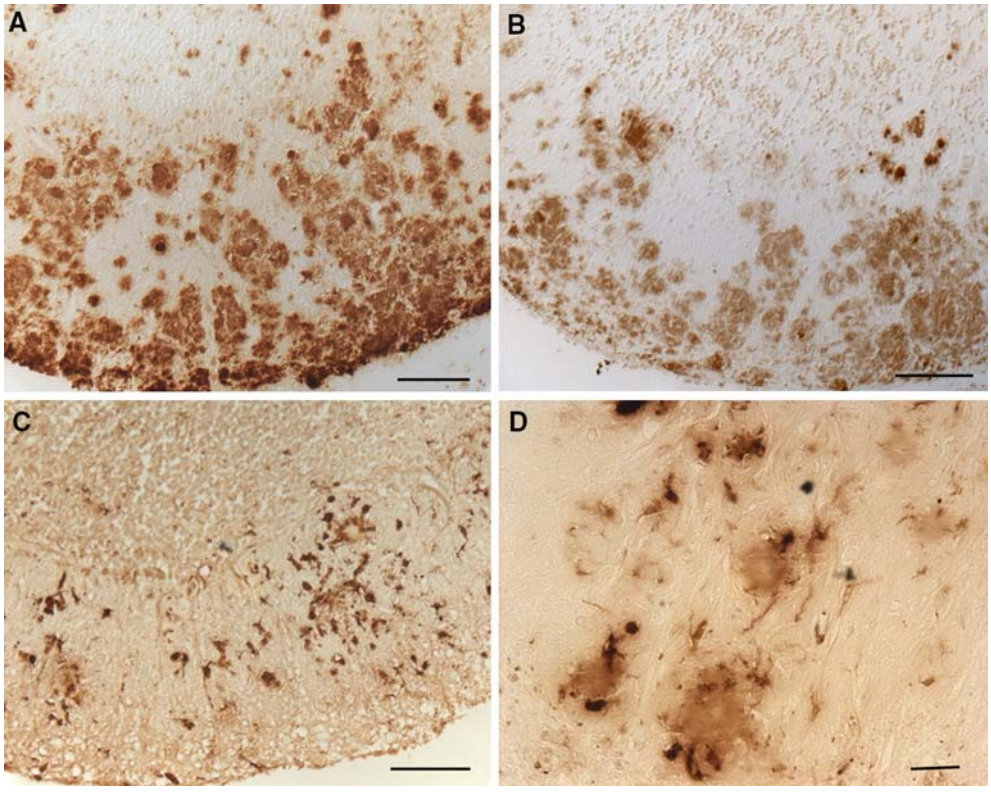

Fig. 1 In GSS cerebellum, prion deposits marked by 3F4 (**A**) also contain high levels of redox-active iron (**B**). Ferritin-positive cells also accumulate around the deposits (**C**). In another GSS case, redox-active iron is also present in cells surrounding PrP deposits (**D**) (*GSS* Gerstmann-Straussler-Scheinker syndrome, *PrP* prion protein). **A**–**C** are serial sections; *bar* 100 μm

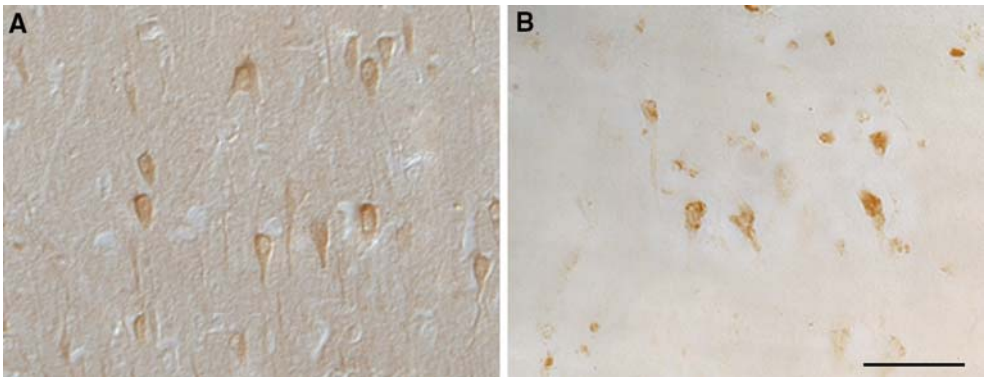

Fig. 2 In GSS in the temporal cortical layers, adjacent to hippocampus, 8OHG (**A**) is elevated in pyramidal neurons as is mtDNA (**B**) (*8OHG* 8-hydoxyguanosine). *Bar* 50 μm

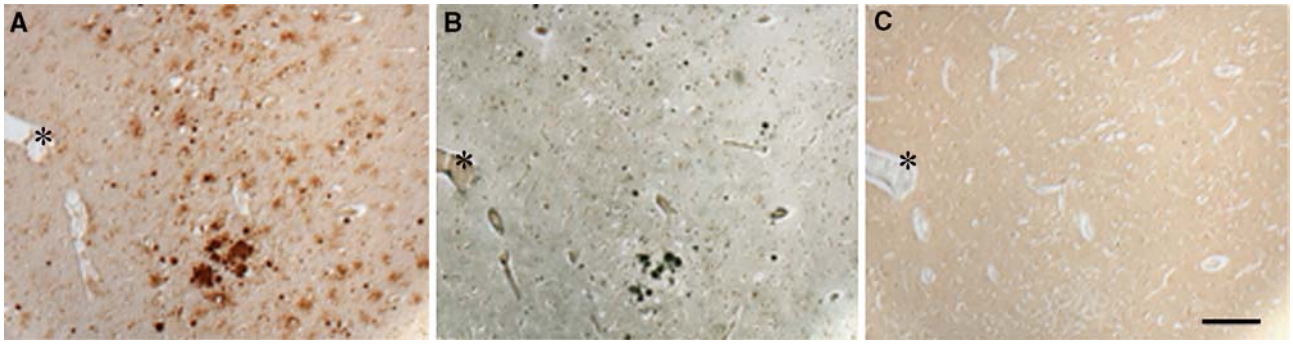

Fig. 3 Adjacent serial sections (landmark vessel shown by *asterisk*) of cortex from a case of vCJD show many cored, dense prion deposits labeled with 3F4 (**A**) contain iron (**B**), but lack amyloid-β (**C**). This profile was found in all three vCJD cases studied (*vCJD* variant Creutzfeld-Jakob disease). *Bar* 100 μm

Variant CJD

In all three cases of vCJD, large numbers of diffuse and focal PrP-containing deposits were seen. The dense focal deposits were positive for redox-active metals, yet lacked amyloid-β (Fig. 3).

Sporadic CJD

Prion deposits in the different cases of sCJD varied morphologically from containing widely dispersed small, thread-like aggregates to having many large defined deposits (plaques). In no case was the redox iron associated with PrP deposits. Four (ages 55, 58, 66, 74 years) out of the 25 sCJD cases studied also contained amyloid-β-positive plaques. These 4G8-positive structures contained redox-active metals (Fig. 4).

A further distinction between the deposits of PrP in sCJD and those of PrP in GSS or amyloid-β in AD was the lack of HO-1 or lipid peroxidation markers such as HNE or CML surrounding the deposits in any of the cases studied, irrespective of fixative (data not shown). In this respect, the results using the methacarn or Carnoy's fixatives, in addition to formalin, are of particular interest since the former usually display oxidative modifications with greater sensitivity [32].

Discussion

In this study, we found that in both young and old cases of GSS, iron and its storage protein, ferritin, are heavily deposited in both the PrP lesions and surrounding cells. Ferritin has recently been shown to be prominently co-transported with protease-resistant PrP [23]. Iron, while always found associated with amyloid-β deposits in AD, is also in PrP in GSS and vCJD, independent of concomitant amyloid-β deposition. PrP deposits in sCJD cases, however, lack iron deposition, further distinguishing this disease from the inherited or infectious forms. This could be due to the shorter disease duration, or less dense, variable PrP accumulations found in sCJD. In this study, metal deposition was only found in association with PrP^{sc} found in amyloid plaques, not with the amorphous deposits typically found in human disease. This implies that the metal deposition either requires the ordered structure found in the plaque, which is not present in the amorphous deposits, or that both arise by processes distinct to iron deposition. Thus, simply possessing a β-sheeted structure does not suffice for metal association. Other markers, such as HO-1, HNE and CML, were found to be slightly increased in GSS and vCJD pathological structures, but were not increased in any case of sCJD. In addition, the pattern

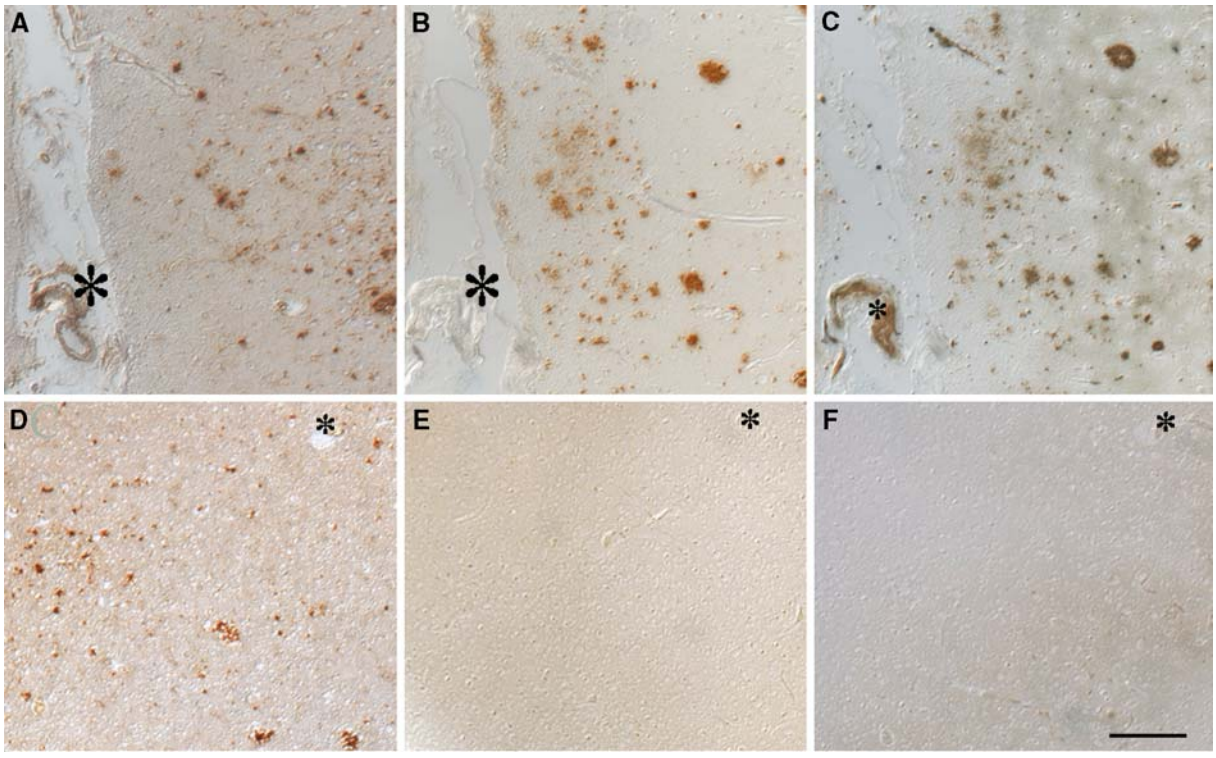

Fig. 4 Cortical sections from a patient with sCJD with PrP deposits (age 66 years) (stained with 3F4, **A**) with concomitant amyloid-β deposition (4G8, **B**). Redox-active metals (**C**) colocalize with only the amyloid-β deposits. Another patient with sCJD (age 36 years), demonstrating aggregates of prion (3F4, **D**), contains neither amyloid-β (**E**), nor redox-active metals (**F**). **A**–**C** and **D**–**F** are adjacent serial cortical sections with landmark vessels (*arrows*) (sCJD sporadic CJD). *Bar* 100 μm

of PrP deposition varied, and this may be one reason why the oxidative damage profile neither compares to the levels found in AD-related lesions nor occurs with regularity, suggesting it is not a prominent feature of lesion formation. The same can also be said for extracellular PrP deposition in sCJD, which occurs with such wide variability among cases, that this feature cannot be essential. Typically, sCJD patients live only months after diagnosis, while GSS and AD both have a disease course lasting many years, possibly suggesting that a protracted disease course is important for protective responses, such as amyloid-β and formation of PrP deposition as plaques, to be induced.

Other neurodegenerative diseases associated with protein amyloid plaques, most notably Alzheimer disease, are also associated with aberrant iron deposits [36]. Such metal ions are redox active [17, 32], and always contribute to increased oxidative stress including lipid peroxidation and HNE adduction [31]. These data, together with the findings presented in this study emphasize the parallels that can be drawn between various neurodegenerative disorders that likely have completely different etiological backdrops [5].

The profiles of oxidative damage and iron deposition found in prion diseases suggests parallels with AD. Early markers of neuronal dysfunction, well characterized in pyramidal neurons in AD, have also been localized in CJD, namely, 8OHG. The pattern of localization was varied, some cases exhibiting global neuronal accumulations, while other cases showed small clusters of positive cells [13]. This pattern was similar to the dense accumulations of apoptotic neurons found in CJD [8]. However, in contrast, 8OHG was found increased to the same extent in pyramidal neurons of the same anatomic area in cases of AD.

PrP has been implicated in maintaining oxidant defenses within cells [30, 39]. Increased PrP expression has been shown to be closely followed by increased antioxidant enzyme and glutathione levels in a cell culture model [29]. Expanding these studies to a scrapie-infected mouse model, it was further noted that PrP^{sc} had a reduced copper-binding capacity, with a proportional decrease in cellular antioxidant levels [37]. Supporting the role of metals in prion disease is the observation that copper chelation delays the onset of prion disease in mice inoculated by an intraperitoneal route [34].

Our findings, combined with previous studies implicate oxidative stress as an important feature of prion diseases, and further suggests that there are important subtypes of oxidative responses in neurodegenerative diseases.

Neuronal loss, additionally, is a ubiquitous feature of both AD and CJD. In AD neuronal loss is highly correlated with dementia. All cases of CJD show demonstrable neuron loss at levels of 45–60% in many areas of the cortex [12]. However, the more striking feature of

CJD is the spongiform change that results from vacuolation in a large percentage of neurons. These neurons would be expected to have impaired function. Consistent with altered metabolism, increased levels of nitrotyrosine and heme-oxygenase in murine studies [13], nucleic acid oxidation [14], and DNA fragmentation [8, 22] were found in CJD.

It may be that β-pleated protein structures coordinate the retention of free redox-active iron, and that loose fibrillar and thread-like prion deposits in sCJD cases may not exhibit the protein structures suitable for iron deposition. The finding of iron deposits in vCJD, with durations similar to sCJD, suggests that it is not simply the duration of disease as might be implied by GSS or iron deposits.

It has been suggested that metal imbalances, resulting from oxidative stress, could be the initiating factor responsible for altering the copper-binding capacity of PrP, which in turn further drives oxidation fluctuations and accumulation of PrP^{sc}. In scrapie-infected neuroblastoma cells, reduced iron metabolism was found [7]. Hall and Edskes [15] have proposed a two-hit model that drives prion disease development, incorporating both the change in protein form and a change in host state in the initiation and progression of disease. This is reflective of an earlier proposal of a two-hit model for AD [41, 42], where it was proposed that both oxidative stress and mitotic signaling pathways are necessary for disease progression. Independently, each factor could initiate disease state; however, it is the combination with an altered cellular environment that propagates disease. Amyloids, including normal PrP, may not retain their neuroprotective functions when their protein form is changed. Therefore, altered conformation combined with an oxidatively challenged environment results in neuronal loss and apoptosis, i.e., neurodegeneration.

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