Neuronal Apoptosis in Creutzfeldt-Jakob Disease

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Abstract. Neuronal loss is a salient feature of prion diseases, however, its causes and mechanisms are unclear. The possibility that it could occur through an apoptotic process has been postulated and is consistent with the lack of inflammation in prion disorders as supported by experimental studies. In order to test this hypothesis in humans, we examined samples of frontal and temporal cerebral cortex, striatum, thalamus, and cerebellum from 16 patients who died from Creutzfeldt-Jakob disease. They included 5 sporadic cases, 5 familial, 3 iatrogenic, and 3 cases with the new variant. These were compared with age and sex matched controls. Using in situ end labelling, we identified apoptotic neurons in all the cases of Creutzfeldt-Jakob disease. A single labelled neuron was found in the eldest control. Apoptotic neurons were mostly found in damaged regions and their presence and abundance seemed to correlate closely with neuronal loss. This supports the view that apoptosis of neurons is a feature of prion diseases and may contribute to the neuronal loss which is one of the main characteristics of these conditions. Neuronal apoptosis also correlated well with microglial activation, as demonstrated by the expression of major histocompatibility complex class II antigens, and axonal damage, as identified by beta-amyloid protein precursor immunostaining. In contrast, we found no obvious relationship between the topography and severity of neuronal apoptosis and the type, topography, and abundance of prion protein deposits as demonstrated by immunocytochemistry.

Key Words: Axonal damage; Creutzfeldt-Jakob disease; Microglial activation; Neuronal apoptosis; Prion diseases.

INTRODUCTION

Neuronal loss, together with spongiosis and astrogliosis, is a salient feature of Creutzfeldt-Jakob disease (CJD) (1). However, its causes and mechanisms are still unclear. The possibility that neuronal loss in prion diseases occurs through a process of programmed cell death has been postulated. Programmed cell death (PCD) is a physiological form of the cell-suicide process, essential in the normal development, maturation, and turnover of tissues. However, abnormal induction of a cell death program may occur in pathological conditions. PCD is an active process, requiring activation signals, signal transduction, and in most instances, gene expression and protein synthesis in the dying cell (2–5). Apoptosis, the phenotype of PCD, differs morphologically and biochemically from necrosis, which is a passive, pathological form of cell death. Morphologically it is characterized by shrinkage of the cell and the nucleus, condensation and fragmentation of the nuclear chromatin, then segmentation of the cell into apoptotic bodies that are rapidly ingested by neighbouring phagocytic cells. Unlike necrosis, in which rupture of the cell membrane leads to release of intracellular toxic enzymes causing an inflammatory reaction, in PCD the plasma membrane is intact with no consequent inflammation in the affected tissue (3, 6–9). Biochemically, apoptosis is characterized by the specific endonuclease-mediated internucleosomal fragmentation into regular subunits multiple of an oligonucleosome length unit of 180 base pairs (10).

The hypothesis that, in CJD, neuronal loss occurs through an apoptotic process might explain the almost complete absence of inflammatory reaction in its pathology. It is also supported by experimental studies. Apoptosis of neurons has been identified in vivo using in situ end labelling (ISEL) and electron microscopy in scrapie infected mice (11). Moreover, the 106–126 synthetic peptide homologous to the prion protein (PrP) has been shown to be neurotoxic and to induce apoptosis of rat hippocampal neurons in cultures (12). These findings have recently been supported in humans, in fatal familial insomnia (FFI) where neuronal loss is the predominant change (13).

The aim of this study was to look for neuronal apoptosis in CJD and to try to correlate its extent and topography with those of PrP deposits, microglial activation, and axonal damage in order to clarify the causes and mechanisms of neuronal damage in this condition.

MATERIALS AND METHODS

In the framework of a European Union Concerted Action on human transmissible spongiform encephalopathies (BMH4-CT97–2034), brain samples from 16 patients who died from CJD were collected. They included 5 cases with sporadic CJD (one of whom also had cerebral amyloid angiopathy (14)); 2 patients with the
E200K mutation; 3 patients with octapeptide repeat inserts (OPRI) (15) (1 with 8 OPRI belonging to the Breton French family [16] already reported by Goldfarb [17] and 2 with 6 OPRI belonging to a recently described Basque French family [18]); 3 patients with iatrogenic CJD (2 who received growth hormone [19] and one with a possible iatrogenic CJD transmitted by pooled plasma derivatives [20]); and 3 patients with the new variant (21) (2 cases from UK and the only French case [22]). Sixteen age and sex matched individuals who died accidentally, without brain injury, collected in a Forensic Medicine Department, were examined according to the same protocol and served as controls. The clinical, epidemiological, genetic, and bibliographic details of these 16 cases are summarized in Table 1.

In each case, we examined at least 5 brain samples including frontal cortex, medial temporal cortex, striatum, thalamus, and cerebellum. Sections from formalin fixed, formic acid postfixed, paraffin embedded specimens were stained with hematoxylin and eosin (H&E). Immunocytochemistry was performed according to the protocol of the CJD Surveillance Unit, Edinburgh (23) with microwave substitution of the autoclaving pretreatment, using the 3F4 antibody (Senetek). Immunohistochemistry was performed, after microwave pretreatment, by an indirect immunoperoxidase method (APAAP) method, using antibodies raised against major histocompatibility class II antigens (HLA-DR), monoclonal, (Dako, 1/100), interleukin-1 (IL-1) (polyclonal, 1/15, Cistron) and tumor necrosis factor-alpha (TNF-α) (polyclonal 1/25, Genzyme) in order to evaluate microglial activation, and against beta-amyloid protein precursor (BAPP) (monoclonal, Boehringer, 1/200) to look for axonal damage. The latter technique can identify early and subtle axonal change (24). In situ end labelling (ISEL) was performed using the Apoptag kit (Oncor) modified as previously described (25) to identify apoptotic neurons. In the initial study (25), we used different methods and compared the results with those of electrophoresis of DNA extracted from frozen cerebral cortex. This technique has been subsequently shown to be a reliable and reproducible method, not influenced by postmortem delay of less than 72 hours, or by formalin fixation of less than 5 weeks (26, 27).

The presence of apoptotic neurons was evaluated semiquantitatively, blindly, by 3 neuropathologists (FG, HAB, FC) and subsequently reviewed jointly, as previously described (25). Briefly, it was scored (0) when no apoptotic neurons were found, (1) when only occasional isolated apoptotic neurons were seen, (2) when occasional nests of apoptotic neurons were observed, and (3) when apoptotic neurons were frequent. Endothelial cells that have a quick turnover and therefore often undergo apoptosis served as positive internal controls. The intensity of IVV; HLA-DR, IL-1β, TNF-α, and βAPP expression was also evaluated semiquantitatively and scored 0 = absent, 1 = mild, 2 = marked, and 3 = intense.

RESULTS

Apoptotic neurons were identified in all the CJD cases (Table 2) whereas only a few labelled neurons were found in the Ammon’s horn of only one control; an 83-year-old woman matched to case 3. Positive in situ end labelling was frequently, but not invariably, associated with morphological changes characteristic of apoptosis with pyknotic nuclei and shrunken cytoplasm (Fig. 1a). In severely affected areas, and frequently in the striatum, apoptotic microglial cells and astrocytes were also present (Fig. 1b).

Apoptotic neurons were identified in affected regions and their distribution and severity seemed to correlate closely with those of the characteristic neuropathological changes: spongiosis, gliosis, and neuronal loss. In contrast, we did not find any obvious relationship between the topography and severity of neuronal apoptosis and the type, topography,
and abundance of PrP deposits as identified by immunocytochemistry. Similarly, within the framework of comparable phenotypes, there was no difference in the abundance and distribution of apoptotic neurons according to the etiology whether sporadic, familial, or iatrogenic of the disease.

Apoptotic neurons were relatively sparse in the cerebral cortex of the new variant cases (cases 14–16) where PrP deposits were massive, and they were usually found at a distance from the deposits. Apoptotic neurons were mostly found in the deeper cortical layers in the cerebral cortex of iatrogenic cases due to growth hormone injection (cases 11, 12), whereas PrP deposits formed plaques predominantly involving the superficial cortical layers. In contrast, neuronal apoptosis had a similar distribution to spongiiform change (Fig. 1a), and was of comparable severity, in the cerebral cortex of sporadic, familial, or iatrogenic cases, whereas PrP deposits were present as plaques or had a synaptic, perivascular, or perineuronal pattern. Apoptotic neurons were particularly abundant in the granular layer of the cerebellum (Fig. 1c) in cases with predominant involvement of the cerebellum whether sporadic (case 2), genetic (cases 9–10), or iatrogenic (cases 11, 13). In these cases, PrP deposits were variable. They were present as plaques or synaptic deposits within the granular layer. However, particularly in cases with OPRI (15), the deposits were mostly, or exclusively present in the molecular layer and formed elongated patches (cases 9, 10) or coalescent plaques (case 8) (Fig. 1d).

Microglial activation, as demonstrated by HLA-DR expression, was constantly identified in CJD cases in affected regions, and its severity correlated well with that of the neuropathological changes i.e. spongiosis, gliosis, and neuronal loss (Table 2). There was frequently a close topographical relationship between activated microglia and the neuropathological changes. Activated microglial cells were observed in close contact with vacuoles (Fig. 2a). Marked HLA-DR expression was also constantly found around and within PrP plaques (Fig. 2b). Interestingly, in many instances, activated macrophages or microglial cells were adjacent to neurons showing the morphological features of apoptosis (Fig. 2c). In contrast, expression of IL-1 and TNF-α was invariant and extremely weak (1) and mainly identified in the cytoplasm of occasional macrophages or microglial cells. No expression of HLA-DR and cytokines was identified in the controls.

Axonal damage was also consistently observed in affected areas, whereas only occasional damaged axons were present in a few controls. Beta-APP positive axonal swellings were present within the lesions, in the vicinity of spongiform change (Fig. 2d), in and around PrP deposits (Fig. 2e, f), and also in the white matter underlying damaged cortex or in pathways efferent from affected nuclei. Positively stained torpedos were also frequent within the granular layer in cases with involvement of the cerebellum and loss of Purkinje cells.

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Fig. 1. (a) Frontal cortex of case 7, ISEL shows an apoptotic neuron (arrow), the nucleus of which is stained in red, within an area of spongiosis. Notice that although the surrounding neurons are morphologically normal, this neuron shows morphological changes characteristic of apoptosis, including pyknotic nucleus and shrunken cytoplasm (ApopTag kit revealed by an APAAP technique; ×400). (b) Striatum of case 2, ISEL shows 2 apoptotic neurons stained in red, within an area of mild spongiosis; 2 glial cells (arrows) are also stained (ApopTag kit revealed by an APAAP technique; ×400). (c) Cerebellum of case 8, ISEL shows 3 apoptotic granules (arrows), the nucleus of which is stained in red, within the granular layer of the cerebellum. Notice that no apoptotic cell is identified in the molecular layer (ApopTag kit revealed by an APAAP technique; ×250). (d) Cerebellum of case 8 (Same case as Fig. 1c). PrP immunostaining shows massive deposits forming confluent plaques, strictly limited to the molecular layer (3F4 antibody (Senetek), ×100).

DISCUSSION

Apoptosis of neurons was identified using in situ end labelling in all our 16 cases with Creutzfeldt-Jakob disease, whereas only a few apoptotic neurons were observed in the Ammon's horn of the eldest control. This supports the view that programmed cell death is a feature of prion diseases as already shown in scrapie infected mice (12, 28, 29) and in human cases with FFI (13), and that it may contribute, at least partly, to the neuronal loss which is a major feature of these conditions. Apoptotic neurons were mostly found in affected brain regions and their distribution and abundance seemed to correlate closely with those of the characteristic neuropathological changes, spongiosis, gliosis, and neuronal loss, and therefore with the phenotype...

Fig. 2. (a) Cerebellum of case 13, HLA-DR immunostaining shows marked microglial activation both in the granular layer and in the molecular layer. In this layer activated microglial cells are present in close contact with vacuoles (APAAP, ×250). (b) Frontal cortex of case 15, HLA-DR immunostaining shows marked expression around and within a characteristic "florid" PrP...
plaque (APAAP, ×1000). (c) Frontal cortex of case 16, HLA-DR immunostaining shows activated microglial cells adjacent to neurons showing the morphological features of apoptosis (arrows) (APAAP, ×250). (d) Thalamus of case 2, βAPP immunostaining shows damaged axons within an area of spongiosis and gliosis (APAAP, ×250). (e) Frontal cortex of case 14, βAPP immunostaining shows marked expression around and within a characteristic "florid" PrP plaque (APAAP, ×1000). (f) Cerebellum of case 8 (Same case as Fig. 1c, d), immunostaining shows axonal damage in the molecular layer, in close contact with PrP plaques (APAAP, ×400).
and severity of the disease. This was particularly striking in the medial temporal cortex where the hippocampus was usually spared and did not show neuronal apoptosis, whereas spongiform changes and apoptotic neurons were frequent in the subiculum.

Axonal swellings expressing βAPP were also constantly observed in affected areas. The etiology of axonal damage is unknown; it is certainly nonspecific and has been described in a variety of pathological processes (30). In CJD, several mechanisms may interact. Axonal damage may result from neuronal loss, as suggested by the observation of frequent bundles of βAPP positive axons in the efferent pathways of the striatum and thalamus in cases with severe involvement of these nuclei. This mechanism is also supported by an immunocytochemical study of synaptophysin in the dorsal lateral geniculate nucleus of mice following intraocular scrapie infection, which showed that reduction of axonal boutons was not seen before neuronal loss was already well established (31). Axonal damage may also be the consequence of PrP deposition. Recent experimental studies, in scrapie infected mice, showed that pre-amyloid PrP accumulation was associated with selective damage to axon terminals and dendritic spines, and that it was a very specific and early process (32). This is consistent with our finding of frequent βAPP positivity within and around PrP deposits (Fig. 2e), as with previous observation by Bugiani et al of degenerative neurites expressing βAPP around PrP deposits in the brain of in patients belonging to the Indiana kindred with Gerstmann-Sträussler-Scheinker disease (33). Finally, axonal damage may result from microglial activation (vide infra).

Microglial activation, as demonstrated by expression of major histocompatibility class II antigens, was also closely related to the other neuropathological changes in distribution and severity, suggesting that together with the astrocytic reaction, it forms a component of the gliosis which is another key neuropathological characteristic of prion diseases. Unlike the situation in neurons, interpretation of positive ISEL staining in glial and microglial cells is not straightforward. As ISEL is not absolutely specific for double-stranded DNA breaks and can also detect single-stranded breaks as observed in cell multiplication (34), positive staining may also reflect cell proliferation. On the other hand, Petito and Roberts suggested that apoptotic death of reactive astrocytes might be a physiological mechanism whereby the brain removes an excess number of astrocytes that have proliferated after certain types of brain injury (27). This can also apply for microglia.

Our observation is consistent with previous studies demonstrating that microglial activation is a feature of human spongiform encephalopathies (35–39). As previously described, marked microglial activation was identified within and around PrP plaques (35) and around vacuoles (37, 39). Activated microglial cells were also frequently observed in close contact with apoptotic cells (Fig. 2c) suggesting 2 possibilities. First, the microglial reaction may just be the consequence of neuronal apoptosis and/or neuronal damage. Transformation of microglia into brain macrophages has been shown to occur in response to several types of injury, including anterograde and retrograde axonal or neuronal lesions (40, 41). It may also be directed toward ingestion of apoptotic neurons (9). Alternatively, microglial activation may play a causative role in neuronal apoptosis and in axonal damage. The differentiation of microglia into brain macrophages is accompanied by the release of cytotoxic mediators such as free oxygen radicals, cytokines, or nitric oxide (NO) (42) and it has been shown that chronic NO exposure produces cell apoptosis (43). Peroxide exposure may also induce axonal damage and spheroids (44). We specifically investigated IL-1 since this cytokine may activate the immunologic form of NO synthase (45); it is also known to upregulate the expression and processing of βAPP (46). However, unlike HLA-DR expression, IL-1β expression and TNF-α expression were weak and inconsistent making this mechanism unlikely. This is in keeping with a recent computerized analysis of lesions combined with immunocytochemistry in human CJD cases, which tends to provide indirect evidence against an early causative involvement of microglia in the development of spongiform change (39). This differs from HIV encephalitis in which production of proinflammatory cytokines is associated with microglial activation (47) and, in our experience, the expression of IL-1 and TNFα matches that of HLA-DR (47, 48). This discrepancy may be explained by the presence of an inflammatory response which may enhance cytokine expression in HIV encephalitis, whereas it is absent in CJD.

The lack of obvious quantitative and/or topographic relationship between neuronal apoptosis and PrP deposits as demonstrated by immunocytochemistry is consistent with the previous observation that neuronal damage in prion diseases does not parallel PrP deposition (49). It is also consistent with an earlier observation by our group of frequent apoptotic neurons (comparable in numbers to what we observed in CJD) in FFI cases who do not show immunocytochemically identifiable PrP deposits (13), although small amounts of PrP∗ have been demonstrated by immunoblotting in restricted brain areas (50).

The mechanism of neuronal apoptosis in CJD, particularly its relationship with the accumulation of the pathogenic protease resistant isoform (PrP∗) of the cellular prion protein (PrP) is still unclear. The lack of a direct association between neuronal damage and PrP∗ deposition may support models of neuropathogenesis based on “loss of function” of PrP∗, such as withdrawal of defined activation signals inducing FCD, rather than neurotoxicity. On the other hand, a number of experimental studies
suggest that PrP\textsuperscript{sc} is neurototoxic (51), and it has been proposed that the dissociation between neuronal damage and the amount of protein only reflects the variability in different brain regions of the timing and rate of accumulation of PrP\textsuperscript{sc} and selective neuronal vulnerability (50, 52). Glutamate-mediated neurotoxicity has been postulated to act through a PCD mechanism (53) and it has been postulated that PrP\textsuperscript{sc} may be neurotoxic through an excitotoxic glutamate-mediated mechanism, since PrP\textsuperscript{sc} associated neuronal death may be blocked by antagonists of the NMDA receptor complex (54). This hypothesis is also consistent with the appearance of the spongiform change, which is possibly secondary to focal excitotoxic degeneration. Finally, neuronal apoptosis might be an indirect consequence of PrP\textsuperscript{sc} deposition. PrP\textsuperscript{sc}-induced dendritic or axonal damage, perhaps enhanced by consequent microglial activation, might contribute to neuronal apoptosis either due to deafferentation (55) or to retrograde neuronal degeneration (56, 57). Such a mechanism would be consistent with the experimental demonstration that PrP\textsuperscript{sc} deposition and microglial activation precede neuronal cell death (29, 51). It would also be consistent with the observation in our cases of familial CJD with OPRI (cases 8–10), of PrP deposits (Fig. 1d) and axonal damage (Fig. 2f) in the cerebellar molecular layer and neuronal apoptosis in the granular layer (Fig. 1c).

ACKNOWLEDGMENTS

The authors wish to thank Professors Cesare, Chazot, Degas, Delaporte, Gérard, Julien, Will, and Doctors Colombier, Créange, and Gentron for providing the clinical data; Drs Capellari, Delaunay-Laupré, Cimberti, Laplanche, and Parchi who provided the genetic information; Professor Darigon and Doctors Guillon and Pariare who examined the control cases in Forensic Medicine; Gisèle Crocket, Isabelle Le Maner, and Véronique Levy for the histological preparation; and Véronique Mathieu for secretarial help.

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Received October 27, 1998
Revision received December 27, 1998
Accepted December 28, 1998