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How do neurons degenerate in prion diseases or transmissible spongiform encephalopathies (TSEs): neuronal autophagy revisited

Paweł P. Liberski[1], **D. Carleton Gajdusek**[2] and **Paul Brown**[3]

[1]Laboratory of Electron Microscopy and Neuropathology, Department of Molecular Biology, Chair of Oncology, Medical Academy Łódź, Poland, [2]Professor Emeritius, [3]Laboratory of Central Nervous System Studies, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, USA

Abstract. As in other neurodegenerative diseases such as Alzheimer's disease, neurons in prion diseases or transmisible spongiform encephalopathies (TSEs) die *via* programmed cell death of which the apoptotic process is relatively well characterized. A subcellular alteration linked to apoptosis is the formation of autophagic vacuoles, which we and others demonstrated in CJD- and scrapie-affected rodent brains. Autophagy may co-exist with apoptosis or may precede it and the process may be induced by apoptotic stimuli. Here, we extend these observations using different model of scrapie and CJD. Both scrapie models (the 263K and 22C-H) demonstrated autophagic vacuoles with the same frequency; hence, they will be described together. While the following changes had been observed simultaneously in different areas of the same sample, this description is organised as if it followed a sequence of events. First, a part of the neuronal cytoplasm was sequestrated by concentric arrays of membrane; that part of the cytoplasm closed by membranes appeared relatively normal but its density often appeared increased. Next, electron density of the central dramatically increased. Then, membranes proliferated within the cytoplasm in a labyrinth-like manner and an area sequestrated by these membranes enlarged and became more complex structure consisting of vacuoles, electron-dense area and areas of normally-looking cytoplasm connected with convoluted membranes. Finally, a large area of the cytoplasm was transformed into a collection of autophagic vacuoles of different sizes. Virtually identical alterations, albeit with much lower frequency, were seen in terminally ill CJD-affected hamsters.

The correspondence should be addressed to P. P. Liberski, Email: ppliber@csk.am.lodz.pl

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INTRODUCTION

As in other neurodegenerative diseases such as Alzheimer's disease, neurons in prion diseases or transmisible spongiform encephalopathies (TSEs) die *via* programmed cell death of which the apoptotic process is relatively well characterized. Indeed, using TUNEL methodology, apoptotic neurons have been repeatedly shown in both naturally occurring and experimentally induced TSEs (Jesionek-Kupnicka et al. 1997, 1999, Dorandeau et al. 1998, Fraser et al.1996, Fraser 2002). At the ultrastructural level, apoptotic neurons are characterized by specific alterations - cell shrinkage, condensation of chromatin and, eventually, formation of so called "apoptotic bodies". Furthermore, some investigators believe that at least a fraction of "dark neurons" may represent those cells undergoing apoptosis. Another subcellular alteration linked to apoptosis is the formation of autophagic vacuoles, which we (Liberski et al. 1992) and others (Boellaard et al. 1989, 1991) demonstrated in CJD- and scrapie-affected rodent brains. Autophagy may co-exist with apoptosis or may precede it and the process may be induced by apoptotic stimuli (Xue et al. 1999). Furthermore, given level of autophagy may define sensitivity of given neuronal population for apoptotic stimuli which may underlie a phenomenon of "selective neuronal vulnerability". Hence, autophagy and apoptosis are interconnected (Bursch et al. 2000).

Cellular autophagy is a physiological degradive process involved, like apoptosis, in embryonic growth and development, cellular remodelling and the biogenesis of some subcellular organelles - viz. multilamellar bodies (Filonova et al. 2000, Hariri et al. 2000, Sattler et al. 2000). Nascent immature autophagic vacuoles coalesce with lysosomes to form degradive autophagic vacuoles. Hence, analogous to apoptosis, only excessive or wrongly placed autophagy bears stigmata of a pathological process. Of note, autophagy is highly enhanced in another brain amyloidosis, Huntington disease, where the signal for autophagy is huntingtin (Kegel et al. 2000). Here, we extend these observations using different model of scrapie and CJD.

METHODS

Animals and strains

Ten hamsters were inoculated with the 263K or 22C-H strains of scrapie (Liberski et al. 1989 a, b). These strains are widely used experimental tools primarily because of relatively short incubation periods which, for mice, ranged from 16 to 18 weeks and for hamsters from 9 to 10 weeks for the 263K strain and 24-26 weeks for the 22C-H strain.

In addition, hamsters were inoculated with the Echigo-1 strain of CJD (Liberski et al. 1997). The Echigo-1 strain of CJD was isolated by Mori and colleagues (1989) from a case of 33 years-old female with a panencephalopathic type of CJD. The inoculum prepared from a CJD patient's brain passaged to guinea pigs with incubation periods (IP) of 728 days at primary and approximately 400 days at subsequent passages. From an animal which exibited active running and an excessive response to external stimuli, a substrain was isolated with substantially reduced IP (254 days). This strain was re-isolated in hamsters with IP of 141 days at the 3rd passage. An incubation period following intracerebral inoculation of hamsters with 10% cleared suspension of the Echigo-1-affected brain was approximately six months. Appropriate control animals were sham-inoculated with saline.

Electron Microscopy

Following intracardiac perfusion with 150 ml of 2.5% glutaraldehyde and 1% paraformaldehyde in cacodylate buffer (pH 7.2), the brains were removed and rinsed in cold fixatives overnight. Samples [1 mm^3] of the right parietal cortex and adjacent corpus callosum were dissected, rinsed in phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated through a graded series of ethanols and propylene oxide and embedded in Embed (Electron Microscopy Sciences, Ft. Washington, PA). Ultrathin sections were stained with lead citrate and uranyl acetate, and specimens were examined using Zeiss 109 or JEM 100 C transmission electron microscopes.

RESULTS

Definition of autophagic vacuoles

Autophagic vacuoles were composed of areas of the cytoplasm sequestrated with single, double or multiple membrane (phagophores) originated from the endoplasmic reticulum. Sequestrated cytoplasm contained ribosomes, occasionally mitochondria, small secondary vacuoles with vesicles, or presented homogenously dense appearance.

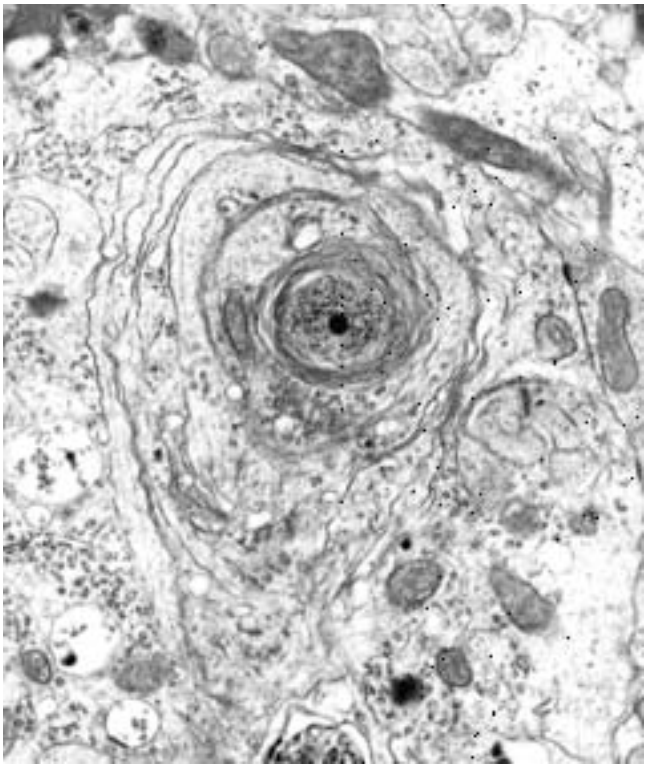


Fig. 1. Some neuronal cytoplasm (circle) sequestrated by a circular array of membranes. Within the central part there is an accumulation of ribosomes and that part appeared denser. The CA2 region of the hippocampus from hamster brain infected with the 263K strain of scrapie. Original magnification, x 12,000.

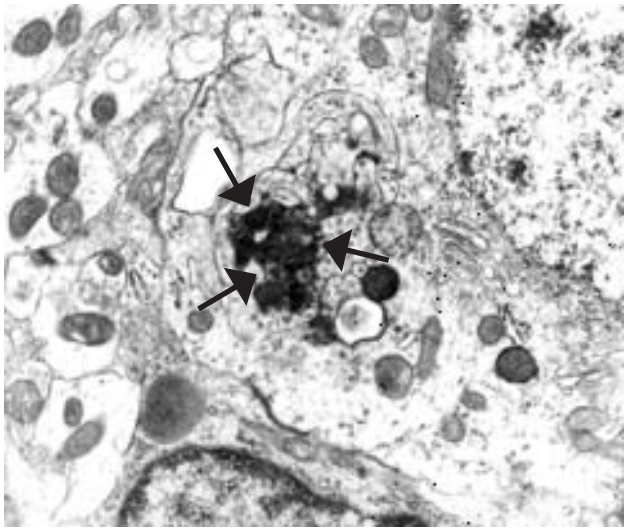
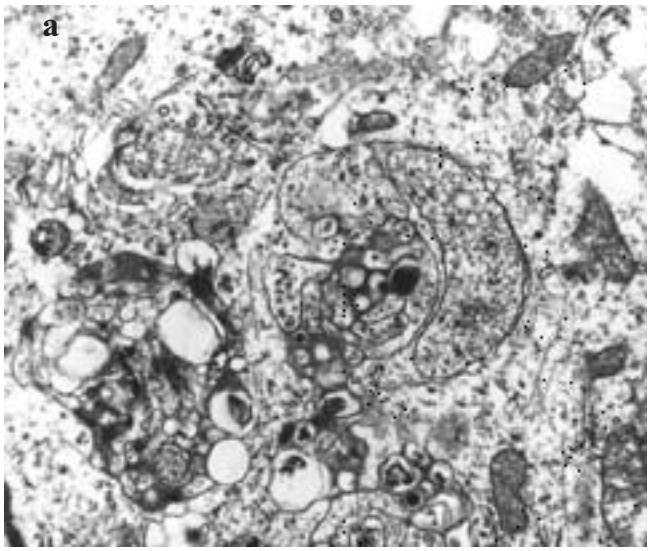


Fig. 2. A situation similar to the previous one but the cytoplasm confined by membranes (arrowheads) exhibits increased density. The CA2 region of the hippocampus from hamster brain infected with the 263K strain of scrapie. Original magnification, x 12,000.

The scrapie-infected hamster brains

Both scrapie models (the 263K and 22C-H) demonstarted autophagic vacuoles with the same frequency; hence, they will be described together. While the following changes had been observed simultaneously in different areas of the same sample, this de-



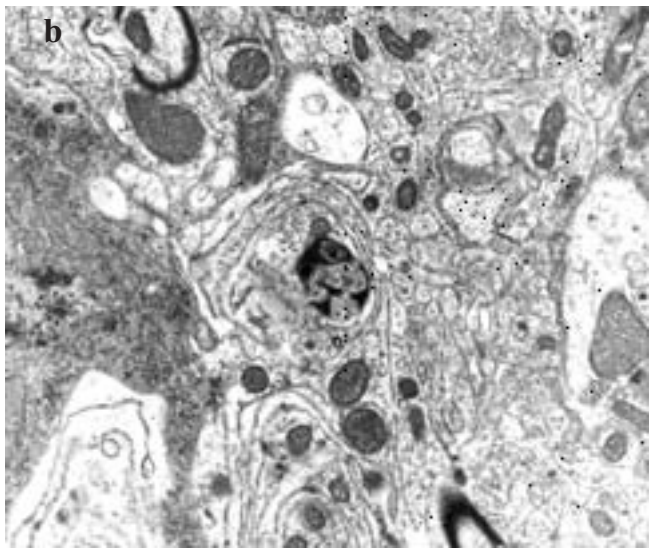


Fig. 3 (a, b). Further development of autophagic vacuoles. Note the complexity of arriving structure with normally-looking parts of the neuronal cytoplasm, areas of different electron density and convoluted membranes sequestrating and connecting other elements. The thalamus below the CA2 region of the hippocampus from hamster brain infected with the 263K strain of scrapie. Original magnification, x 12,000.

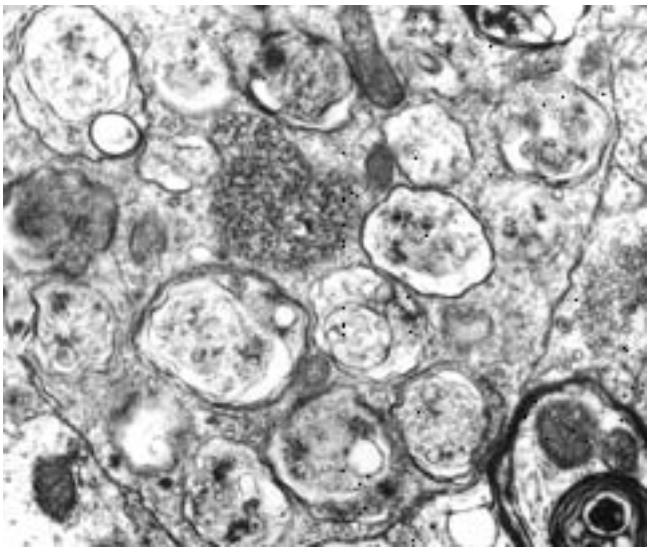


Fig. 4. A large area of the neuronal cytoplasm is replaced by a collection of autophagic vacuoles. The CA2 region of the hippocampus from hamster brain infected with the 263K strain of scrapie. Original magnification, x 30,000.

scription is organised as if it followed a sequence of events. First, a part of the neuronal cytoplasm was sequestrated by concentric arrays of membrane; that part of the cytoplasm closed by membranes appeared relatively normal but its density often appeared increased (Fig. 1). Next, electron density of the central part dramatically increased (Fig. 2). Then, membranes proliferated within the cytoplasm in a labyrinth-like manner and an area sequestrated by these membranes enlarged and became more complex structure consisting of vacuoles, electron-dense area and areas of normally-looking cytoplasm connected with convoluted membranes (Fig. 3). Finally, a large areas of the cytoplasm was transformed into a collection of autophagic vacuoles of different sizes (Fig. 4)

The CJD-affected hamster brain

Virtually identical alterations, albeit with much lower frequency, were seen in terminally ill CJD-affected hamsters (data not shown).

DISCUSSION

One of the major problems of TSEs pathogenesis is a final cause of neuronal degeneration with eventual neuronal loss (Fraser 2002). The basic event which underlies pathology (vacuoles and astroglial proliferation) and most probably causes it, is conversion of a normal isoform of prion protein (PrP^{c}) into its pathological isoform (PrP^{Sc}) (Weissmann et al. 1996, Prusiner 2001, Collinge 2001, Peretz et al. 2002) with a parallel change from α-helical into β-pleated sheath (or β-helical) secondary structure of PrP. Indeed, using a highly sophisticated mathematical model, Stumpf and Krakauer (2000) tried to answer whether PrP engenders neurons to die because of neurotoxic effect of PrP^{sc} (gain of function) or loss of function of PrP^{c}. They assume that if cells die of apoptosis because of neurotoxic gain of function of PrP^{Sc}, the amount of PrP^{Sc} is low because those cells die fast. Indeed in both CJD and fatal familial insomnia (FFI), there is more apoptotic cells and lower amount of PrP-amyloid (Dorandeau et al. 1998) than, for instance, in Gerstmann-Sträussler-Scheinker disease (GSS), where amount of amyloid is vast and number of apoptotic cells low (Gray et al. 1999).

There are conflicting data that neurons, and perhaps glia, in TSEs die of apoptosis. Migheli et al. (1995) found no evidence of apoptosis in scrapie-infected mouse brains. To the contrary, Giese et al. (1995); Lucassen et al. (1995), Fraser et al. (1996) Williams et al. (1997), Kretzschmar et al. (1996, 1997, 1998) and Jamieson et al. (2001) found apoptosis in different rodent scrapie models readily. The characteristic DNA fragmentation ladder have been seen in BSE (Theil et al. 1999) and in natural scrapie (Fairbarin et al. 1994) while *in situ* end-labeling (ISEL) method revealed apoptotic cells in human and experimental CJD (Gray et al. 1999, Jesionek-Kupnicka et al. 1999, Ferrer 1999). Collectively, these data strongly suggest that neurons die in TSE because of apoptosis.

It is evident that PrP accumulation in TSE-affected brain precedes development of other changes - viz. spongiform change and astrogliosis for which PrP serves as a signal for proliferation (Brown 1998, 1999, Brown et al. 1998, Ye et al. 1998, Hafiz and Brown 2000). At the ultrastructural level, apparently normal looking neurons secrete PrP which later on fibrilizes and as such becomes neuro- or neuronotoxic (Jeffrey et al. 2000). The latter euphemism describes total lack of knowledge as to the sequence of events which leads to neuronal decay is completely unknown. What is known, is the end-stage - apoptosis. However, some *in vitro* experiments highlight the possible intermediate steps. Several synthetic peptides which form amyloid fibrils viz. PrP106-126 (Brown 2000) or PrP118-135 (Pillot et al. 2000) induce apoptosis in a dose-dependent manner.

PrP106-126 may exert its pro-apoptotic characteristics *via* disruption of mitochondrial membrane with a subsequent release of cytochrome-c and caspase activation (O'Donovan et al. 2001). ext, intracellular Ca^{2+} concentration raises and another family of proteases, calpains are activated. To the contrary, Thellung et al. (2000) found that intracellular Ca^{2+} concentration is decreased *in vitro* in rat granule cells following treatment with PrP106-126 and such an effect is mimicked by nicardipine, the L-type voltage-sensitive calcium channel blocker. Taken into account the protean nature of PrP peptides, even whether they adopt α–helical or β–pleated conformation, these conflicting data are not that surprising. Even more discordant data were presented by Bounhar et al. (2001) who found that PrP may serve as an anti-apoptotic factor protecting neurons *in vitro* from Bax-induced apoptosis. Removal of four of five octarepeats (codon 51 - 91 of PrP) or D178N and T183A *PRNP* mutations completely abolished these effect. It is immediately apparent that gain of function of PrP (in PrP^{Sc}) may result in changing of anti-apoptotic into pro-apoptotic properties of PrP which is loosely reminiscent changes of anti-oncogenic into oncogenic function of p53 protein (also involved in apoptosis).

However, even such a simple correlation between PrP, pathology and final neuronal drop out may not be entirely clear. Following transmission of BSE from cattle to mice, Lasmezas et al. (1997) found two lines of mice, both infected with BSE, one with PrP and typical TSE pathology (vacuolation and astrogliosis) and a second, without PrP but prominent apoptosis of neurons. In independent experiment, the highest density of apoptotic cells have been observed in those neuroanatomical areas in which spongiform changes are minimal or absent - viz. retina and the cerebellum (Kretzschmar et al. 1996). Thus, apoptosis and PrP may not only be uncoupled under specific experimental conditions but may be directly (not mediated through PrP) linked to scrapie infectivity.

Neurons may only degenerate and die through limited number of pathologic pathways. The cellular necrosis is a well known one but totally unspecific. It is caused by a sudden brain insult; leads to destruction of the whole cells the remnants of which attract inflammatory cells. Apoptosis is unspecific also but it is programmed molecular process following a "suicidal" stimulus which leads to a hierarchical gene response. Apoptotic, in contrast to necrotic cells, attract no inflammatory response. As the relative lack of classical immunological response in TSE-affected brain is paradygmatic for the whole group of these diseases (Brown 1990), neurons, by definition, should die by apoptosis and not necrosis.

The problem with visualisation of apoptotic cells is based on the time over which neurons die. Even when the number of neurons in dorsal lateral geniculate nucleus (dLGN) following intraocular inoculation at the opposite site drops from ca 22,000 to less than 2,000 (Jeffrey et al. 1995), the number of apoptotic nuclei detected by TUNEL method is low (Fraser et al. 1996). As a result, apoptotic cells are most readily detected in highly structured neuronal systems, retina and hippocampus are the best examples. As a result what is observable may present a minute proportion of all apoptotic event going on in TSE-affected brains.

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